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The Secretary of Health, Education, and Welfare has determined that the publication of this periodical is necessary in the transaction of the public business required by law of this Department. Use of funds for printing this periodical has been approved by the Director of the Office of Management and Budget through November 30, 1976.

Eight-Drug Combination Chemotherapy (MOPP and ABDV) and Local Radiotherapy for Advanced Hodgkin's Disease[1,2,3]

Delvyn C. Case, Jr, Charles W. Young,* Lourdes Nisce, Burton J. Lee III, and Bayard D. Clarkson[4]

SUMMARY

Thirty-seven patients with advanced Hodgkin's disease have been treated for ≥ 3 months with a protocol consisting of alternate monthly courses of MOPP (mechlorethamine, Oncovin [vincristine], procarbazine, and prednisone) and ABDV (adriamycin, bleomycin, DTIC, and vinblastine) with local radiotherapy (RT) to areas of originally bulky disease. This therapy produced CR in 19 of 19 previously untreated patients (100%), eight of nine previously treated with RT (89%), and six of nine previously treated with RT and MOPP (67%). The remaining patients are all PRs tending toward CR status. The median time to CR was 3.0 months. The median time in remission to date for the previously untreated patients is 8+ months (2+–14+). After an induction period of eight cycles of chemotherapy patients are maintained on alternate-month treatment continuing the alternating sequence. During this phase three patients have experienced reappearance of disease (one recurrence, one possible second primary lymphoma, and one recurrence in a patient whose original diagnosis is in doubt). The regimen has been well tolerated. All patients were treated as outpatients. Alopecia and neurotoxicity were mild and myelosuppression was moderate. Clinically significant cardiopulmonary toxicity has been limited to mild radiation pneumonitis in one patient and bleomycin pneumonitis which cleared during prednisone in a second patient.

[Cancer Treat Rep 60:1217–1223, 1976]

The MOPP (mechlorethamine, Oncovin [vincristine], procarbazine, and prednisone) regimen has become the most widely used chemotherapeutic program in previously untreated patients with advanced Hodgkin's disease (1–4). Between 70% and 80% of the patients enter CR during treatment with six to nine cycles of this treatment; the reported median duration of unmaintained remission has ranged from 15 to > 36 months. Although some MOPP-treated patients remain in unmaintained CR for many years, 40%–60% of the patients eventually relapse (1–5). The value of maintenance therapy after MOPP induction has not yet been established (2,5,6). Several modifications of MOPP have been tested in previously untreated patients in the hope of improving both the incidence and duration of CR (7,8). None of these has shown therapeutic superiority over MOPP.

Recently, Bonadonna et al (9,10) reported the results of a therapeutic trial with a four-drug protocol incorporating adriamycin, bleomycin, DTIC, and vinblastine (ABVD) for remission induction of previously untreated patients with advanced Hodgkin's disease. Each of these drugs produces an objective response in 40%–60% of such patients (11–13). In a prospective randomized comparison, Bonadonna observed CR in 19 of 25 patients (76%) treated with MOPP and in 15 of 20 patients (75%) treated with ABVD (10). In three patients resistant to MOPP, treatment with ABVD produced one CR and one

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[2]Presented in part at the 12th annual meeting of the American Society of Clinical Oncology, Toronto, Ontario, Canada, May 4–8, 1976.

[3]Supported in part by Public Health Service grants CA-08748 and CA-05826 from the National Cancer Institute, National Institutes of Health, Department of Health, Education, and Welfare.

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PR. In a series of 24 MOPP-resistant patients treated at Memorial Hospital, a modified version of the Bonadonna program, ABDV, produced a significant response (CR + PR) in 63% of the patients (14). These studies suggest that cross resistance between the two regimens may be absent in a significant number of patients. The availability of two effective non-cross-resistant regimens makes possible the construction of treatment programs using both of them in the hope of preventing late relapse.

In constructing the actual regimen we have also made use of recent studies which have shown significant improvement in disease-free survival in patients receiving combined chemotherapy and radiotherapy (RT) (15–17). The selection of ports was influenced by the observation of Frei et al (2) who observed an excellent correlation between the largest pretreatment of tumors in Hodgkin's disease and the site of initial recurrence after MOPP therapy. Chemotherapeutic regimens combined with RT to these bulky areas might decrease local recurrence in the high-risk areas.

Because of the impaired immune response in Hodgkin's disease patients and the immunosuppressive effects of therapy, the addition of immune adjuvants to combined-modality regimens is gaining currency. We have chosen levamisole for this purpose; it is a widely used antihelmintic drug which appears to improve host-defense mechanisms by stimulating phagocytes and lymphocytes when their functions are depressed. Preliminary data on the effect of this drug in lung and breast cancer in man are encouraging (18,19). Recently, the administration of levamisole to patients with Hodgkin's disease has been reported to convert them from skin-test negative to skin-test positive and raise their E-rosette-forming cell number (T cells) from low to normal levels (20,21).

In this paper, we report the preliminary results of remission induction with this eight-drug protocol (MOPP/ABDV) in sequential monthly cycles combined with RT to the areas of originally bulky disease. The objectives of this study are: (*a*) to improve the CR induction rate in patients with advanced Hodgkin's disease; (*b*) to increase the median duration of remission and survival using two effective regimens without cross-resistance; (*c*) to reduce the relapse rate in bulky areas by adding local RT to the chemotherapeutic regimen; and (*d*) to randomly test whether the addition of levamisole, an immune potentiator, can prolong the remission duration once CR is attained.

PATIENTS AND METHODS

Patients

A total of 45 patients were entered in this study between January 1975 and January 1976. Presently, 37 patients are considered evaluable for the analysis of remission induction. Patients with advanced Hodgkin's disease (IIB, IIIB, and IV [A and B]) were eligible for this study who were in the following categories: (*a*) previously untreated, (*b*) previously treated with RT, and (*c*) previously treated with RT and chemotherapy (MOPP) relapsing after unmaintained remission of > 12 months. Extent of disease workup was carried out according to present conventions. Staging determinations were made in accordance with the Ann Arbor classification (22). The distribution of patients by age, sex, histologic type, stage, and prior therapy is presented in table 1. The Lukes and Butler classification was used (23).

TABLE 1.—Clinical data of evaluable patients

	Prior therapy		
	None	RT	RT and chemotherapy
No. of patients	19	9	9
Mean age (yrs)	33 (16–56)	27 (19–47)	32 (21–44)
Sex			
Men	9	4	5
Women	10	5	4
Histology			
Lymphocyte predominance	1	1	0
Nodular sclerosis	14	6	3
Mixed cellularity	4	2	6
Stage			
IIB	3	3	0
IIIB	7	2	3
IV, A and B	9	4	6

Treatment Program

The timing and doses of the eight-drug chemotherapeutic program are shown in figure 1. The sequence of the combined therapy is shown in table 2. MOPP was administered in the first, third, sixth, and eighth month. Prednisone was given only in the first and fourth cycle of administration of MOPP (ie, first and eighth month). A single-dose limit of 10 mg of mechlorethamine, 2 mg of vincristine, and 150 mg of procarbazine was observed. ABDV was administered in monthly cycles in the second, fourth, seventh, and ninth month with modifications of the original Bonadonna et al regimen (9,10) as previously described (14). Bleomycin (2 mg in 0.2 ml) was injected by deep sc administration (at the maximal depth of a No. 25G 5/8-inch needle perpendicular to the skin) on Days 4–12 and 18–26. DTIC (250 mg/m² iv) was given on Days 1 and 15. [In the Bonadonna ABVD program, bleomycin (10 mg/m² iv) was given on Days 1 and 15 and DTIC (150 mg/m²/day) was given on Days 1–5 (10).] In the present study, a single-dose limit of 10 mg of vinblastine was observed. Adriamycin was omitted in the 13th, 17th, and 21st month in those patients who had received mediastinal RT as part of this protocol or in those patients who had received mediastinal RT in a previous regimen. The maximal total dose of adriamycin in this patient group was 200 mg/m². The attenuation schedule is presented in table 3. All patients were treated and managed as outpatients.

RT to areas of originally bulky nodal disease (> 5 cm in diameter) was given in the fifth month to a tumor dose of 2000 rads in 2 weeks. After a 2-week rest period chemotherapy was resumed. In no patient was extensive RT given to the liver or lungs even when those organs were clearly involved by Hodgkin's disease.

In the tenth month patients were randomized to receive, or not to receive, levamisole in the months between chemotherapeutic courses on the maintenance part of the protocol (table 2). Levamisole was given at a dose of 150 mg/day orally × 6 days on Days 2–7 and 16–21 and in the tenth, 12th, 14th, 16th, 18th, 20th, 22nd, and 24th–30th month.

MOPP/ABDV/RT - ADVANCED HODGKIN'S DISEASE
DOSAGE SEQUENCE

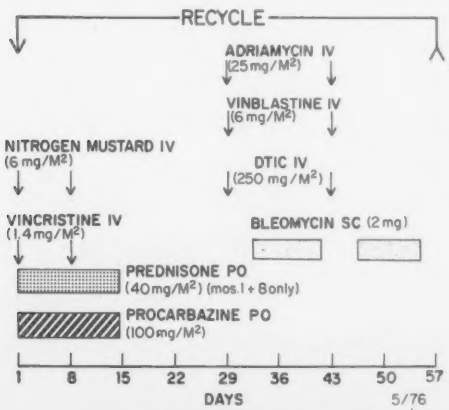
RECYCLE
ADRIAMYCIN IV (25 mg/M²)
VINBLASTINE IV (6 mg/M²)
NITROGEN MUSTARD IV (6 mg/M²)
DTIC IV (250 mg/M²)
VINCRISTINE IV (1.4 mg/M²)
BLEOMYCIN SC (2 mg)
PREDNISONE PO (40 mg/M²) (mos. 1 + 8 only)
PROCARBAZINE PO (100 mg/M²)
1 8 15 22 29 36 43 50 57
DAYS
5/76

FIGURE 1.—Drug timing and doses.

TABLE 2.—Scheduling of MOPP, ABDV, and RT

Month		Month	
1	MOPP	14	Rest or levamisole
2	ABDV	15	Mechlorethamine, vincristine, and procarbazine
3	Mechlorethamine, vincristine, and procarbazine	16	Rest or levamisole
4	ABDV	17	Bleomycin, DTIC, and vinblastine*
5	RT†	18	Rest or levamisole
6	Mechlorethamine, vincristine, and procarbazine	19	Mechlorethamine, vincristine, and procarbazine
7	ABDV	20	Rest or levamisole
8	MOPP	21	Bleomycin, DTIC, and vinblastine*
9	ABDV	22	Rest or levamisole
10	Rest or levamisole	23	Mechlorethamine, vincristine, and procarbazine
11	Mechlorethamine, vincristine, and procarbazine	24–30	Rest or levamisole
12	Rest or levamisole		
13	Bleomycin, DTIC, and vinblastine*		

*Adriamycin was not given if mediastinal RT was given in the 5th month or if there was a history of prior mediastinal RT.
†2000 rads given to areas of originally bulky disease.

Assessment of Results

A CR was defined as the absence of all symptoms and physical, X-ray, scan, and biochemical signs attributed to Hodgkin's disease with peripheral blood counts that were normal or showed only the effects of cytotoxic drugs. PR was defined as the disappearance in constitutional signs and symptoms and ≥ 50% reduction of the longest perpendicular diameter of all evidence of disease. At least 1 month in remission was required to be considered a response. An adequate trial was defined as 3 months of treatment.

RESULTS

The results of the remission induction of this protocol are shown in table 4. In the previously untreated group, 19 of 19 patients (100%) achieved a

TABLE 3.—Attenuation schedule

Wbc count (cells/mm³)	Platelet count (platelets/mm³)	
> 4000	> 130,000	Full doses
3000–3999	100,000–120,000	100% (vincristine, prednisone, bleomycin, and DTIC) 50% (mechlorethamine, procarbazine, adriamycin, and vinblastine)
2000–2999	50,000–99,000	100% (vincristine, prednisone, and bleomycin) 50% (DTIC) 25% (mechlorethamine, procarbazine, adriamycin, and vinblastine)
1500–1999		100% (bleomycin and prednisone) 50% (vincristine) 25% (mechlorethamine, procarbazine, DTIC, and vinblastine; no adriamycin)

CR. Prior RT did not affect the response rate; in this group eight of nine patients (89%) achieved a CR; the patient with a PR is tending toward CR. In the group previously treated with RT and MOPP, six of nine patients (67%) achieved a CR with the remaining three patients in PR tending toward a CR. The median time required to achieve a CR was 3 months. The median duration of remission to date is 8+ months (2+–14+ months) in the untreated group, 13+ months (2.5+–15+ months) in the RT group, and 7+–15+ months (1.5+–16+ months) in the group previously treated with RT and MOPP.

The toxic manifestations are reported in table 5. All patients were able to be treated as outpatients. Administration of mechlorethamine and DTIC was followed by severe nausea and vomiting without significant relief by injectable antiemetics. There was significant alopecia from adriamycin and paresthesias from vincristine and vinblastine. In three patients a substantial reduction of the dose of vincristine was necessary because of severe paresthesias, neurotoxicity, and ileus; amenorrhea occurred infrequently.

Bone marrow suppression represented the main dose-limiting factor. The incidence of transient leukopenia, as determined on the day of drug administration, was the same after MOPP as after ABDV. Only one patient with MOPP/ABDV showed a wbc count of < 2000 cells/mm³. Thrombocytopenia occurred in the minority of patients; in no instance was the platelet count < 80,000 platelets/mm³. Incidence and degree of myelosuppression were not significantly different between the patients previously treated with RT and the patients previously treated with RT and chemotherapy. Drug-related infection was observed in only two patients, one of whom received an excessive dose of vinblastine through error.

One patient developed transient radiation pneumonitis and another patient developed interstitial pneumonia secondary to bleomycin; both responded to prednisone. No clinical cardiomyopathy secondary to adriamycin has occurred. The maximum total dose for those patients with prior mediastinal RT is set at 200 mg/m² because of the synergistic effect on cardiac toxicity between adriamycin and RT (24). Those patients who do not receive RT on this protocol and who have not received mediastinal RT in previous regimens are scheduled to receive a total dose of 350 mg/m². The total dose of bleomycin on this protocol is 250 mg. Patients are followed with routine chest roentgenograms to monitor any evidence of cardiopulmonary toxicity. Electrocardiograms and systolic time interval determinations will be done if symptoms of toxicity develop in these patients.

Three patients have developed reactivation of the neoplastic process during the maintenance-therapy phase. One patient developed disease in a cervical lymph node, a previously uninvolved site, while otherwise remaining in CR and continuing on main-

TABLE 4.—Response rates

	Prior therapy		
	None	RT	RT and chemotherapy
Responses			
CR	19 (100%)	8 (89%)	6 (67%)
PR	0	1*	3*
Failures	0	0	0
Median duration of remission: May 1, 1976	8+ (2+–14+)	13+ (2.5+–15.5+)	7+–15+ (1.5+–16+)
Median time to CR (mos)	3	1.5	3

*Early responders, tending toward CR status.

TABLE 5.—Toxic manifestations in 37 patients

Effect	Frequency
Leukopenia (wbc count < 4000 cells/mm³)	
MOPP	42%
ABDV	49%
Thrombocytopenia (platelet count < 130,000 platelets/mm³)	
MOPP	6%
ABDV	6%
Paresthesias	75%
Alopecia	75%
Amenorrhea	2/13 (menstruating women)
RT or bleomycin pneumonitis	1 patient

tenance therapy. The node was excised and read as diffuse poorly differentiated lymphocytic lymphoma with plasmacytoid features. Review of the original slides confirmed the diagnosis of Hodgkin's disease with mixed cellularity. A repeat extent of the disease workup revealed no other evidence of disease. Whether this represents a second primary lymphoma or a recrudescence of Hodgkin's disease in an altered histologic pattern cannot presently be determined. The patient had been in CR for 8 months.

The second patient had a cutaneous lymphoma without nodal or visceral involvement which presented difficulty in histologic interpretation. The best available interpretation was Hodgkin's disease. During maintenance-phase therapy, after 9 months in CR, a cutaneous recurrence developed which is less suggestive of Hodgkin's disease. Although the diagnosis remains in doubt the clinical behavior now favors mycosis fungoides or a diffuse histiocytic lymphoma.

After 8 months in CR a third patient relapsed in an axillary node which was not irradiated although originally bulky through a gap in protocol observance. This patient, who exceeded 2 m² in body surface area, at no time experienced significant count depression and may have been under-treated. In view of this we have dropped the arbitrary dosage limits on mechlorethamine, vincristine, procarbazine, and vinblastine originally written into the protocol.

Table 6 presents the percentage of optimal dose per cycle of each medication during the first 8 months of chemotherapy. The percentage of optimal dose per cycle of MOPP and ABDV remained approximately the same during the 4 months of treatment with these drugs. In most cases when a reduction in dosage was required, it occurred during the second half of a monthly cycle because of the dropping blood cell count produced by the first doses of medication. Usually by the time of recycling, the counts had returned to normal values. Rather than delaying treatment, appropriate attenuated doses of medication were used on the scheduled treatment day.

DISCUSSION

In this study we have combined MOPP and ABDV sequentially in alternating monthly cycles in an attempt to improve the induction of CR and remission duration. This approach of using two effective non-cross-resistant drug combinations was first anticipated by DeVita and later suggested by Bonadonna et al. RT is added to treat locally the areas of originally bulky disease. This rationale is based upon the relapse pattern described by Frei et al (2), wherein an excellent correlation existed between the site of the largest tumor in Hodgkin's disease and the site of initial recurrence. The use of moderate-dose RT (2000 rads) is based upon the hypothesis

TABLE 6.—Percentage of optimal dose per cycle

					Average total course
	Month—				
MOPP	*1*	*3*	*6*	*8*	
Mechlorethamine + procarbazine	90%	90%	80%	80%	85%
Vincristine	95%	95%	95%	95%	95%
Prednisone	100%			90%	95%
	Month—				
ABDV	*2*	*4*	*7*	*9*	
Adriamycin + vinblastine	72%	65%	70%	72%	70%
DTIC	80%	80%	72%	72%	76%
Bleomycin	95%	100%	100%	100%	99%

that the optimum tissue dose of RT required to control local disease can be reduced when RT is combined with multiple-drug chemotherapy. Drug therapy can reduce the cells in the grossly involved area to 10^4–10^5 cells. The residual cells then can be sterilized with RT in the dose range of 1500–2500 rads (17). This lower dose also allows for further optimal dosage of chemotherapy after RT is concluded.

To date in this study, 19 of 19 previously untreated patients (100%), eight of nine previously treated with RT (89%), and six of nine previously treated with RT and MOPP (67%) have achieved a CR. The median time to CR (3 months) compares favorably with reported data for MOPP (3.5 months) and ABVD (4.5 months) when given in a randomized series. The majority of patients achieved CR status prior to receiving RT. The exceptions were patients with massive adenopathy and a nodular sclerosing histology. This protocol is easy to administer on an outpatient basis. Myelosuppression has been moderate and without serious consequences. The nausea and vomiting after mechlorethamine and DTIC have been troublesome but these symptoms usually last only 12–18 hours. In five patients it has been necessary to shift from mechlorethamine to thio-TEPA (15–18 mg/m^2) because of intolerable vomiting. Alopecia has been less severe than expected. Cardiac toxicity from adriamycin and RT has not as yet been observed but the time of followup is short. Because most patients in the previously untreated group achieved a CR before the RT month, smaller ports of RT could be used, thereby minimizing toxicity to the heart and lungs.

The induction regimen in this protocol offers an effective and rapid means of inducing a CR in patients with advanced Hodgkin's disease in both previously untreated patients and in previously treated patients who are not multiple-drug resistant. In view of the high remission rates obtained with either MOPP or ABDV alone, the excellent response to their use in alternate sequence is not surprising. The critical yardstick that will determine the value of this therapeutic approach must be the duration of remission and, hopefully, the prevention of late relapse. Years of further observation will be required to determine whether or not this protocol is superior to the other well-studied regimens.

The potential for long-term treatment-related complications in this program deserves serious consideration. Cardiopulmonary toxicity from adriamycin and bleomycin can be increased when these agents are used in conjunction with RT (24–26). In addition, the risk of developing second primary neoplasms may prove significant in protocols combining RT and chemotherapy (27–29). Accordingly, for the present, it must be considered a meritorious investigative approach but not conventional management.

LIST OF COMPOUNDS

Adriamycin: NSC-123127; CAS reg. No. 23214-92-8
Bleomycin: NSC-125066; CAS reg. No. 11056-06-7; [2,4′-bithiazole]-4-carboxylic acid, 2′-(2-aminoethyl)-, monohydrate
DTIC: NSC-45388; CAS reg. No. 4342-03-4; imidazole-4-carboxamide, 5-(3,3-dimethyl-1-triazeno)-
Levamisole: NSC-177023; CAS reg. No. 16595-80-5; imidazo[2,1-*b*]thiazole, 2,3,5,6-tetrahydro-6-phenyl-, monohydrochloride
Mechlorethamine: NSC-762; CAS reg. No. 55-86-7; diethylamine, 2,2′-dichloro-*N*-methyl-, hydrochloride
Prednisone: NSC-10023; CAS reg. No. 53-03-2; pregna-1,4-diene-3,11,20-trione, 17,21-dihydroxy-
Procarbazine: NSC-77213; CAS reg. No. 491-88-3; *p*-toluamide, *N*-isopropyl-α-(2-methylhydrazino)-, monohydrochloride
Vinblastine: NSC-49842; CAS reg. No. 6449-03-2; vincaleukoblastine, sulfate (1:1), monohydrate
Vincristine: NSC-67574; CAS reg. No. 2068-78-2; leurocristine, sulfate (1:1)

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Cyclophosphamide, Adriamycin, Methotrexate, and Procarbazine (CAMP)—Effective Four-Drug Combination Chemotherapy for Metastatic Non-Oat Cell Bronchogenic Carcinoma[1,2]

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SUMMARY

Twenty-three patients with metastatic non-oat cell bronchogenic carcinoma (MNOBC) were treated with cyclophosphamide, adriamycin, methotrexate, and procarbazine (CAMP) after radiation therapy. Objective responses were seen in 11 of 23 patients (48%) with a projected median survival time of 12.5 months for responding patients and patients with stable disease. Lung, liver, and cutaneous sites of involvement proved to be highly responsive; bone involvement was less responsive. CAMP is an effective combination in the treatment of MNOBC and its use in patients with less advanced disease should be explored.

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Carcinoma of the lung is still one of the most lethal and untreatable human solid tumors. The median survival for patients with untreated metastatic non-oat cell bronchogenic carcinoma (MNOBC) is only 4–5 months (1). Radiation therapy given even at high dosages only extends survival for these patients by 1–2 months (2). There have been recent reports on drug regimens which suggest that some slight progress has been made in the treatment of this disease (3–5). Our encouraging experience with a four-drug combination of cyclophosphamide, adriamycin, methotrexate, and procarbazine (CAMP) after radiation therapy for the treatment of MNOBC is the subject of this report.

MATERIALS AND METHODS

From January 1975 to January 1976, 26 patients with MNOBC (stage III_{M1}, Carr and Mountain [6]) were referred for entry into a pilot chemotherapy program either at the University of Chicago Hospitals or at the Michael Reese Hospital in Illinois. All patients required histologic proof of malignancy. The cell types consisted of 14 squamous cell carcinomas, six adenocarcinomas, four large-cell undifferentiated carcinomas, and two bronchoalveolar carcinomas.

All patients had clinical evidence of metastatic disease which was documented by scintigraphic studies (liver, bone, and brain scans), by radiographic studies (chest roentgenogram or bone survey), or by biopsy. After the presence of metastatic disease was established, 19 patients received radiotherapy to the lungs (3000–4500 rads in ten to 15 fractions) because of primary lung lesions that exceeded 4 cm, and three patients received radiation therapy to brain metastases (3000 rads in ten fractions). Four patients received no radiotherapy. Radiotherapy was followed by chemotherapy within 1

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TABLE 1.—CAMP combination chemotherapy

Drug	Dose (mg/m^2)	Schedule	Route of administration
Cyclophosphamide	300	Days 1,8	Iv push
Adriamycin	20	Days 1,8	Iv push
Methotrexate	15	Days 1,8	Iv push
Procarbazine	100	Days 1–10	Oral

or 2 weeks which consisted of the CAMP combination shown in table 1 given on an outpatient basis.

Chemotherapy was discontinued during Days 11–28. Chest roentgenograms were taken on a monthly to bimonthly schedule. Liver, bone, and brain scans were performed every 3–4 months. Cbc and platelet counts were done on a weekly basis for determination of the nadirs.

The drug dose was modified according to wbc and platelet counts:

Wbcs		*Platelets*	*% of dose given*
> 4000	and	> 100,000	100
< 4000 > 3000	and	> 100,000	75
< 3000 > 2000	and	< 100,000 > 75,000	50
< 2000	and	< 75,000	0

Adriamycin was administered up to a total dose of 450 mg/m^2. When this dose level was achieved, chemotherapy was continued with cyclophosphamide, methotrexate, and procarbazine.

The Karnofsky index for the determination of performance status was used prior to the initiation of and during chemotherapy (7).

RESPONSE

Any lesion that had been irradiated was classified as an unevaluable parameter of disease because radiation fibrosis often obscured the primary lung tumor making response difficult to assess. Nonetheless, if there was enlargement of an irradiated lung lesion, the patient was considered to have disease progression. Liver involvement observed by physical examination required a 30% reduction in span to be considered a response. Liver involvement documented by scan required complete disappearance of nodules to be considered a response; a slight reduction or no change in the appearance of the liver scan was considered stable disease.

Complete response (CR) is defined as the disappearance of all clinical evidence of tumors. Partial response (PR) is defined as a reduction of > 50% of the sum products of the greatest tumor diameter and its perpendicular for all measurable lesions for ≥ 2 months. Stable disease (SD) is defined as no progression or a < 50% reduction for ≥ 2 months, and was not included as an objective response. Progressive disease or the appearance of new lesions is considered no response (NR). Since many patients had bone disease, this parameter was evaluated as follows: if the bone scan demonstrated a reduction in the number of metastatic lesions, the patient was considered as having a PR. If the bone scan showed no progression, the patient was considered to have SD. If progressive disease was seen on the bone scan, the patient was considered as having NR. Since few lytic lesions show objective evidence of healing on bone survey, the bone survey was not used as a parameter. Similarly, osteoblastic bone lesions were considered nonevaluable.

Patients who received radiation to brain lesions were considered as having SD if they had no other evaluable parameters of disease. Survival was calculated from Day 1 of chemotherapy; for patients to be considered evaluable, it was necessary that they complete at least one cycle of chemotherapy.

RESULTS

Of the 26 patients entered, 23 completed at least one cycle of chemotherapy (table 2). Three patients were excluded because of early death during irradiation of the primary lung lesion (one patient) or refusal to receive any further chemotherapy (two patients) after the first injection of the CAMP combination.

The response rate according to histology is shown in table 3. Among the 23 evaluable patients, responses were seen in 11 of 23 (48%). Two patients had SD. One patient received radiation therapy to the primary lung lesion and brain metastasis. The other patient received radiation therapy to the lung primary; her bone lesion showed no progression on the bone scan.

The response rate for individual sites of involvement is shown in table 4. The liver proved to be a highly responsive site (71%), as did the contralateral lung and skin (70%). Bone, on the other hand, proved to be resistant, with four of 16 patients having objective responses (25%) as documented by bone scan. No patient with lytic bone lesions found on bone survey showed objective evidence of healing.

The high objective response rate, 11 of 23, seen in our patients treated with CAMP may be related to their performance status since most of our patients had a high Karnofsky index (mean, 65). However, if

TABLE 2.—University of Chicago lung cancer pilot study (stage III_{MI})

Age (yrs)	Sex	Histology	Performance status (initial)	Metastatic sites	Response		Survival* (mos)
					Type	Duration (mos)	
Responders and stable disease patients							
45	M	Bronchoalveolar	80	Skin, liver	CR	9+	9+
61	F	Bronchoalveolar	80	Lungs, lymph nodes	PR	4+	4+
77	M	Adenocarcinoma	60	Skin, bone	PR	7	7−
47	M	Adenocarcinoma	40	Skin, bone	PR	11+	11+
68	M	Adenocarcinoma	80	Lungs	PR	10+	10+
62	M	Large cell	40	Lungs	CR	4+	4+
59	M	Squamous cell	80	Skin, bone	PR	7+	7+
62	M	Squamous cell	70	Liver, bone	PR	8	12−
60	F	Squamous cell	80	Bone	SD	13+	13+
51	M	Squamous cell	60	Brain	SD	8	9−
63	M	Squamous cell	70	Liver	CR	10†	10−
68	F	Squamous cell	80	Liver, bone	CR	15+	15+
60	M	Squamous cell	60	Liver, bone	PR	5	6−
Nonresponders							
57	F	Adenocarcinoma	80	Bone	NR		4−
64	M	Adenocarcinoma	40	Bone, brain	NR		3+
47	M	Adenocarcinoma	40	Bone, liver	NR		3−
75	M	Large cell	50	Bone, brain	NR		3−
51	M	Squamous cell	80	Lungs	NR		3−
47	F	Squamous cell	60	Lungs, bone	NR		1−
68	M	Squamous cell	70	Liver, bone	NR		7−
62	M	Squamous cell	70	Lungs, bone	NR		5−
60	M	Squamous cell	70	Bone	NR		4−
59	M	Squamous cell	40	Bone	NR		1−

*Calculated from Day 1 of chemotherapy. + = continued survival; − = dead.
†Death secondary to sepsis while patient was still in remission; no autopsy performed.

TABLE 3.—Response rate by histologic stratification in evaluable patients

Histology	No. of patients responding/No. treated (%)	Response type (No. of patients)		
		CR	PR	SD
Squamous cell	5/13 (39)	2	3	2
Adenocarcinoma	3/6 (50)		3	
Bronchoalveolar	2/2 (100)	1	1	
Large cell, undifferentiated	1/2 (50)	1	—	—
Totals	11/23 (48)	4	7	2

one examines the response rate stratified by performance status, seven of 12 patients (59%) with a Karnofsky index ≥ 70 had objective responses, and four of ten (40%) with a Karnofsky index ≤ 60 had objective responses. Thus, performance status had little role in predicting response in an individual patient.

SURVIVAL

Survival by the actuarial method is shown in figure 1. The median survival for the entire group of 23 patients is 8 months. The median survival for the ten nonresponding patients is 3.1 months. Eight of 13 patients (CR + PR + SD) are still alive, with a projected median survival of 12.5 months. There is a highly significant difference in survival between these patients (CR + PR + SD) and nonresponding patients ($P < 0.01$, generalized Wilcoxon). The range of survival is 4–15+ months.

DRUG DOSAGE AND TOXICITY

The average dose per each course of chemotherapy administered was 81% of the calculated dose for

TABLE 4.—Response rate by site of involvement

Metastatic site	No. of patients responding/No. treated (%)	Response type (No. of patients)			
		CR	PR	NR	SD
Liver	5/7 (71)	3	2	2	
Bone	4/16 (25)		4	11	1
Contralateral lung or skin	7/10 (70)	3	4	3	

cyclophosphamide, adriamycin, and procarbazine and 90% for methotrexate.

Toxic side effects are shown in table 5. The median nadir for the total granulocytes was 1.5×10^3 and occurred between Days 17 and 21. The median nadir for the platelet count was approximately 155 $\times 10^3$, also at Days 17–21. Two patients were unable to tolerate procarbazine-induced nausea and vomiting and had to discontinue the drug. There was only one febrile episode that required hospitalization in the entire group of 23 patients; this resulted in a drug-related death, secondary to sepsis.

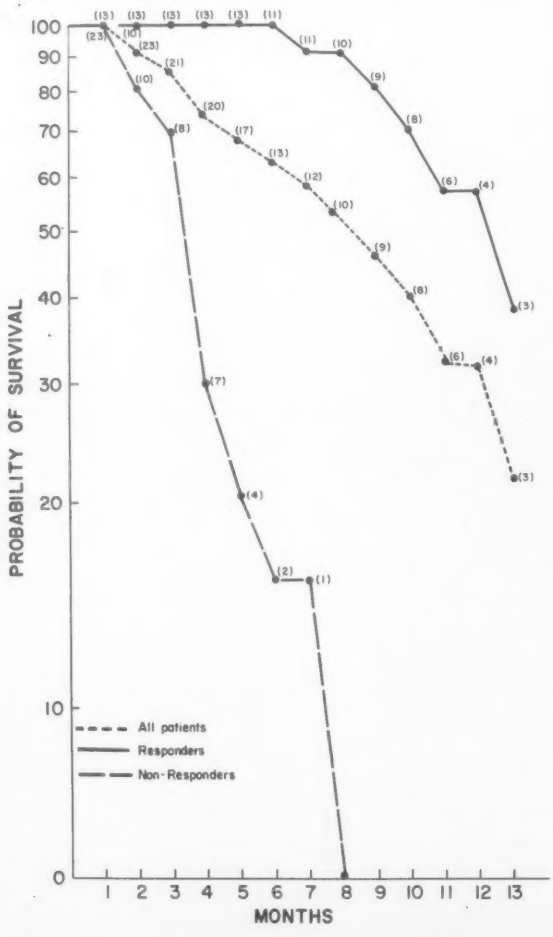

FIGURE 1.—Actuarial survival of 23 patients with MNOBC treated with CAMP; responders' curve includes the 2 patients with SD (*see* table 2).

DISCUSSION

The principle of combination chemotherapy is to combine non-cross-resistant drugs that are effective individually in the treatment of a given tumor and do not have similar toxic effects. This principle is employed in the CAMP combination. Each drug individually has been shown to be effective in the treatment of MNOBC (9). Indeed, the effect of this combination appears to be in excess of the observed objective response rate for each of the individual drugs (15%–20%).

In the drug treatment of any tumor, chemotherapeutic agents must show benefit by both increasing survival and maintaining an acceptable quality of survival. Previously reported drug combinations have led to little increase in survival, as shown in table 6, except for the bleomycin, adriamycin, CCNU, vincristine (Oncovin), and mechlorethamine (nitrogen mustard) (BACON) combination which gave a significantly longer survival in MNOBC (6 months in responders compared to 2 months for nonresponders). Cyclophosphamide, vincristine (Oncovin), methyl-CCNU, and bleomycin (COMB) (3) and BACON (5) are toxic regimens that cause

TABLE 5.—Toxic effects of CAMP

Effect	
	No. of patients (total treated, 23)
Alopecia	23
Nausea and vomiting	23
Fever	1
	Median No. of cells $\times 10^3$/mm^3 (range)
Hematologic	
Total granulocytes	1.5 (0.3–2.1)
Total platelets	155 (50–650)

TABLE 6.—Survival data in MNOBC

Regimen	Response rate (%)	Median survival of responders (mos)	Ref No.
Cyclophosphamide, methotrexate, actinomycin D, vincristine	—	4	8
Bleomycin, cyclophosphamide, vincristine, methotrexate, 5-fluorouracil	39	4	4
COMB	33	3	3
BACON	50	6	5
CAMP	48	12.5	Present study

marked myelosuppression. With the COMB regimen (3), 20% of all patients treated required hospitalization for administration of antibiotics because of fever. Sixty-seven percent of the patients treated with the COMB regimen and 44% of patients treated with the COMB-variant regimens (3) had absolute granulocyte counts of < 500 cells/mm^3. Furthermore, patients treated with the COMB regimen had a "debilitation syndrome" characterized by anorexia, nausea, weight loss, and apathy. BACON (5) was associated with leukopenia and fever in 16% of patients treated; however, there was only one drug-related death due to sepsis.

The CAMP combination is much less myelosuppressive than BACON or COMB. There was only one episode of sepsis in the 23 treated patients. The most common side effect of this regimen is the nausea and vomiting caused by the daily dose of procarbazine, which required discontinuation of this drug in two of 23 patients.

Radiation therapy alone or followed by single-agent chemotherapy has done little to increase significantly survival in patients with bronchogenic carcinoma who have extensive disease (2,9). Our patient population had evidence of metastatic disease at the onset of drug therapy. While it is difficult to assess the role that radiation therapy had in increasing survival, it is clear that the CAMP combination caused objective responses in metastatic lesions in 11 of 23 patients and stabilization of disease in an additional two patients. The projected survival of these patients is well in excess of that reported with radiation therapy alone (2,9) and probably reflects the beneficial effects of the CAMP combination in increasing survival.

CAMP is an effective beneficial drug combination in patients with MNOBC, and its use in larger clinical trials, with patients having less advanced disease, should be explored.

LIST OF COMPOUNDS

Actinomycin D: NSC-3053

Adriamycin: NSC-123127; CAS reg. No. 23214-92-8

BACON = bleomycin, adriamycin, CCNU, vincristine (Oncovin), and mechlorethamine (nitrogen mustard)

Bleomycin: NSC-125066; [2,4′-bithiazole]-4-carboxylic acid, 2′-(2-aminoethyl)-, monohydrate

CAMP = cyclophosphamide, adriamycin, methotrexate, and procarbazine

CCNU: NSC-79037; CAS reg. No. 13010-47-4; urea, 1-(2-chloroethyl)-3-cyclohexyl- 1-nitroso-

COMB = cyclophosphamide, vincristine (Oncovin), methyl-CCNU, and bleomycin

Cyclophosphamide: NSC-26271; CAS reg. No. 6055-19-2; 2*H*-1,3,2-oxazaphosphorine, 2-[bis(2-chloroethyl)amino]tetrahydro-, 2-oxide, monohydrate

Mechlorethamine: NSC-762; CAS reg. No. 55-86-7; diethylamine, 2,2′-dichloro-*N*-methyl-, hydrochloride

Methotrexate: NSC-740; CAS reg. No. 59-05-2; glutamic acid, *N*-[*p*-[[(2,4-diamino-6-pteridinyl)methyl]methylamino]benzoyl]-

Methyl-CCNU: NSC-95441; CAS reg. No. 33073-59-5; urea, 1-(2-chloroethyl)-3-(4-methylcyclohexyl)-1-nitroso-

Procarbazine: NSC-77213; CAS reg. No. 491-88-3; *p*-toluamide, *N*-isopropyl-α-(2-methylhydrazino)-, monohydrochloride

Vincristine: NSC-67574; CAS reg. No. 2068-78-2; leurocristine, sulfate (1:1)

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Sequential Laparoscopy and Laparotomy Combined With Bone Marrow Biopsy in Staging Hodgkin's Disease[1]

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SUMMARY

In a series of 121 unselected, previously untreated patients with Hodgkin's disease staging laparoscopy combined with needle bone marrow biopsy detected the presence of extranodal disease in the liver or marrow or both in 9% of the patients. A spleen biopsy yielded positive findings in 13%. Subsequent laparotomy with open marrow biopsy performed in 110 patients with negative liver and marrow findings from the first combined procedure revealed the presence of extranodal hepatic lymphoma in two additional patients with systemic symptoms and splenic lymphoma in 26 additional spleens. Surgical marrow biopsy was always interpreted as negative. Laparoscopy produced a lesser incidence of morbidity compared to laparotomy. Although devoid of major complications, biopsy of the spleen is not recommended as a routine procedure in staging laparoscopy. This prospective sequential study confirms that laparoscopy plus needle marrow biopsy is a useful, rapid, safe, and economic procedure to establish stage IV disease in the large majority of patients with nodal involvement. Considering the recent more extensive use of chemotherapy for intermediate stages of Hodgkin's disease, our findings suggest that laparotomy with splenectomy needs a critical re-evaluation as a routine staging procedure for patients with no overt extranodal lymphoma.

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In 1969, after Glatstein et al (1) presented their first report on the value of diagnostic laparotomy in Hodgkin's disease, medical oncologists, radiotherapists, and hematologists were immediately divided into two groups of thought. The first group felt that the procedure should be reserved only for selected groups of patients (2) while the second group gradually became convinced that in low-risk patients with no overt extranodal disease, laparotomy with splenectomy and multiple-tissue biopsies (including open iliac crest marrow biopsy) had to be regarded as the best available method to properly stage Hodgkin's disease. During the Ann Arbor Conference (3) the results of the initial experience were responsible for the modification of the stage classification by identifying the splenic involvement within the group of stage III patients (III_S), and for introducing the concept of clinical stage (CS) and of pathologic stage (PS). During the same conference, DeVita et al (4) reported their first series of patients staged by laparoscopy. By examining the results obtained after taking six liver biopsies (three from each lobe), they stated that laparoscopy could represent a valid alternative diagnostic procedure to laparotomy in establishing the presence or absence of hepatic lymphoma. The results were subsequently updated in 47 patients and the conclusion remained unchanged (5).

The controversy between laparoscopy and laparotomy was somewhat revitalized by two recent papers (6,7) in which the relative merits of both procedures combined with needle and open marrow biopsies respectively were sequentially studied. In 65 untreated patients six of seven with liver or marrow disease or both were shown by Spinelli et al (6) to have extranodal Hodgkin's disease in these

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sites by laparoscopy plus needle marrow biopsy. Among 26 previously treated patients this finding occurred in six of 11 patients. Furthermore, spleen biopsies obtained during laparoscopy detected infiltration by lymphoma in 14 of 37 patients (38%) with involved spleen. Spinelli's conclusion was that all patients should be subjected to laparoscopy plus needle marrow biopsy before undergoing staging laparotomy. The results of the comparative study of Vinciguerra et al (7) were in line with our findings since only one of 23 patients had liver involvement at laparotomy after a negative laparoscopy.

The purpose of this paper is, through updating our series of untreated patients, to stress that there is a need to re-evaluate the role of staging laparotomy as a routine procedure in view of the consistent findings of laparoscopy and of the recent more extensive use of chemotherapy for Hodgkin's disease.

PATIENTS AND METHODS

The series includes 121 unselected, previously untreated patients admitted to the Institute from October 1973 to February 1976. The patient ages ranged between 13 and 69 years. The histopathologic diagnosis was established by a biopsy of cervical, axillary, or mediastinal adenopathy. The slides of both initial and abdominal biopsies were reviewed. The patients were clinically staged according to the criteria adopted at Ann Arbor. In particular, bipedal lymphography was carried out in all patients. Liver and spleen scans were performed only when it was thought that they could add further information to define the CS more accurately.

A unilateral Jamshidi needle marrow biopsy was obtained from the posterior iliac crest 1–3 days before performing laparoscopy: this latter surgical procedure was carried out in all patients without knowing the results of marrow biopsy, following the technique previously described (6). In particular, one to six biopsies (average, three biopsies) were obtained from the liver and, whenever technically feasible, one to three biopsies were also obtained from the spleen. When there were no tumor nodules on the surface, blind biopsies of both organs were performed. Staging laparotomy and unilateral open iliac crest marrow biopsy were performed only in patients with negative histologic findings in the liver on laparoscopy and in the bone marrow on needle biopsy. Marrow biopsy was obtained by using an electric saw which yields a specimen of approximately 2 × 2.5 cm in diameter.

RESULTS

Table 1 presents the results of laparoscopy plus needle marrow biopsy. In 11 patients the spleen was not sufficiently visible to obtain a specimen while in two other patients samples were not adequate for histopathologic examination. The incidence of hepatic and marrow involvement was the same (6%) and in three patients biopsies showed a concomitant infiltration of both extranodal sites. There was spleen involvement in 13% of the patients in whom biopsy was feasible and adequate. Thus, the combined procedure modified the CS in a total of 21% of the patients. It is important to note that only three of 17 patients classified as stage IV because of hepatic involvement according to the Ann Arbor criteria were found to have Hodgkin's disease in the liver after biopsy.

TABLE 1.—Liver and spleen findings by laparoscopy and bone marrow findings by needle biopsy*

CS	Total No. of patients	Positive findings in—†		
		Liver	Spleen	Bone marrow
I	34	1/34	3/29‡	0/34
II–II_E	46	0/46	3/41	0/46
III–III_E	23	3/23	4/23	3/23
III_S	1	0/1	1/1	0/1
IV§	17	3/17	3/14	4/17
Totals	121	7/121 (6%)	14/108 (13%)	7/121 (6%)

*11 of 121 patients (9%) had extranodal disease; 26 of 121 patients (21%) had stage modification.

†No. of patients with positive findings/No. tested.

‡No. of patients with adequate sample.

§Staged for liver involvement only.

TABLE 2.—Laparotomy plus open marrow biopsy findings in patients pathologically staged with laparoscopy and needle marrow biopsy*

After laparoscopy + needle marrow biopsy		Patients with positive findings after laparotomy + open marrow biopsy		
CS	No. of patients	Liver	Spleen	Marrow
I	32	1†	6	0
II, II_E	47	0	8	0
III, III_E	20	0	12	0
III_S	11	1†	11	0
Totals	110	2	37	0

*2 of 110 patients (2%) with extranodal disease; 27 of 110 patients (25%) with stage modification.
†Patient had systemic symptoms.

TABLE 3.—Clinical vs pathologic staging*

	Clinical staging		Pathologic staging			
			Laparoscopy + needle marrow biopsy		Laparotomy + open marrow biopsy	
Stage	No. of patients	%	No. of patients	%	No. of patients	%
I	34	28	32	26	26	24
II, II_E	46	38	47	39	39	35
III, III_E	23	19	20	17	8	7
III_S	1	1	11	9	35	32
IV	17	14	11	9	2	2
Totals	121		121		110	

*13 of 121 patients (11%) with extranodal disease.

The results of laparotomy plus open marrow biopsy in 110 patients with negative findings in the liver after laparoscopy and in the marrow after needle biopsy are reported in table 2. Hepatic involvement on wedge biopsy was detected in two patients with PSs IB and III_SB after the first surgical staging procedure. The number of biopsies performed during laparoscopy in the false-negative patients was two and five respectively. It is important to note that after both surgical procedures no patient with stage II disease was found to have liver involvement. Splenectomy confirmed the positive findings observed after laparoscopy in the 11 patients with PS III_S and detected occult splenic involvement in 26 additional patients (24%). Contrary to what has been previously reported by Rosenberg (8), open bone marrow biopsy failed to detect in this particular series additional findings in patients with negative needle samples. Laparotomy as a secondary surgical staging procedure revealed occult extranodal disease in only 2% of the patients. By excluding patients with PS III_S after laparoscopy, laparotomy modified the stage in 25% of the patients.

It is noteworthy that during this study no false-negative or false-positive findings were observed in sampled abdominal nodes. In particular, no positive samples were found in the porta hepatis, nor in the celiac or mesenteric lymph nodes. Although lymph nodes of the porta hepatis were explored in all patients, biopsies were performed in only 32% of the patients.

A comparative evaluation of CS versus the two types of PS is shown in table 3. The first combined procedure established the presence of Hodgkin's disease in the liver and/or marrow in most patients (11 of 13) with extranodal involvement, and contributed to the evidence of splenic lymphoma in about one third of the patients with spleen involvement. The second diagnostic procedure was mainly responsible for a more complete definition of stage III_S since only two false-negative findings were detected in extranodal sites.

Table 4 outlines the pattern of association with spleen, liver, and marrow involvement. As previously reported and despite the fact that in this series most of the stage IV patients were detected by laparoscopy, there was a high correlation between liver and/or marrow disease and spleen involvement. Table 5 also confirms a known finding, showing that the incidence of both splenic and extranodal lymphoma was highest in patients with mixed cellularity and nodular sclerosis.

Morbidity from both surgical procedures is listed in table 6. The most important complication of laparoscopy was one episode of hepatic bleeding which required emergency laparotomy. In two patients laparoscopy was followed by a temporary hemoglobin fall > 2 g but the site of bleeding could not be determined. In another patient a large asymptomatic clot under the splenic capsule was detected at the time of staging laparotomy. In one patient a temporary increase of serum bilirubin (3.2 mg/100 ml) and serum transaminases (SGOT 120 units/ml, SGPT 185 units/ml) was attributed to the trauma produced by multiple liver punctures. Laparotomy with splenectomy produced the expected incidence of morbidity as reported in our prior series (9,10). The most important complication was a small intestinal occlusion. This required a second laparotomy for lysis of adhesions after 3, 6, and 7 months respectively from the surgical staging procedure. Altogether, this comparative evaluation confirms that laparoscopy produced a lesser morbidity than laparotomy.

TABLE 4.—Pattern of association with spleen, liver, and marrow involvement

	No. of patients	Staging by laparoscopy	Staging by laparotomy
Spleen alone	35*	11	25
Liver alone	3	3	
Marrow alone	4	4	
Liver + marrow	1	1	
Spleen + liver	3*	1	2
Spleen + liver + marrow	2	2	

*1 patient determined to have spleen involvement at laparoscopy was found to have liver involvement at laparotomy.

TABLE 5.—Correlation between histologic subgroups and splenic or extranodal disease

	Total No. of patients	Spleen positive only		Liver and/or marrow positive (± spleen positive)	
		No. of patients	%	No. of patients	%
Lymphocytic predominance	20	3	15	2	10
Nodular sclerosis	53	13	25	5	9
Mixed cellularity	28	16	57	5	18
Lymphocytic depletion	20	3	15	1	5
Totals	121	35		13	

TABLE 6.—Complications from laparoscopy and laparotomy

Complication	Laparoscopy (121 patients)	Laparotomy (110 patients)
None	117 (97%)	69 (63%)
Abdominal bleeding—	3 (2.4%)	
From liver	1*	
Site not determined	2	
Atelectasis		3
Bronchopneumonia		13 (31%)
Bronchopneumonia + pleural effusion		18
Hepatitis (traumatic ?)	1	
Pancreatitis		1
Wound infection		2
Intestinal occlusion		3
Wound hemorrhage		1

*1 patient required emergency laparotomy.

DISCUSSION

Using a larger series of untreated patients the present study confirms our initial findings about the relative merits of staging laparoscopy and laparotomy combined with needle and open bone marrow biopsy respectively (6). The first combined procedure detected Hodgkin's disease in the liver in the large majority of patients (seven of nine) with extranodal involvement of this site. It is important to note that the false-negative findings from laparoscopy occurred in patients with systemic symptoms for

whom, as discussed later, chemotherapy is indicated. Laparoscopy was also able to establish the presence of splenic lymphoma in about one third of the patients with spleen involvement. Furthermore, the findings observed in this series suggest that bone marrow biopsy with a Jamshidi needle can probably substitute in clinical practice for open surgical technique. As recently pointed out by Brunning et al (11), bilateral needle biopsies have increased the yield of positive marrow samples. Since the procedure produces minimal discomfort to the patients and because of the focal nature of bone marrow involvement of Hodgkin's disease, two trephine biopsies can be recommended, especially in patients with B symptoms, to ensure a more accurate diagnosis. Our data, which confirm previous reports from the NCI (4,5) and appear consistent with two studies performed in previously untreated patients, suggest that there is a need for a critical re-evaluation of the use of laparotomy as a routine staging procedure in patients with no overt stage IV disease. The reassessment of the role of laparotomy in Hodgkin's disease appears particularly important in view of the recent successful results of mechlorethamine, vincristine, prednisone, and procarbazine (MOPP) chemotherapy in stages II and III (12), and because at present, new combined treatment with radiotherapy and chemotherapy is being applied to many patients with early and intermediate stages.

The advocates of staging laparotomy (1,13) as well as the Committee on Hodgkin's Disease Staging Procedures at Ann Arbor (14) stated that this surgical procedure should be performed only if management decisions depend on the histologic identification of abdominal disease. In particular, it was felt important to determine the splenic status surgically since the spleen appears to be the most difficult abdominal organ to assess on clinical grounds alone and because of the prognostic implications of splenic involvement (15–17). There are no doubts that laparotomy with splenectomy has provided an unparalleled contribution to the understanding of the natural history of Hodgkin's disease. More importantly from a practical point of view, through accurate staging patients with occult abdominal disease have received more adequate treatment programs which ultimately led to improved survival (18).

The major points to be discussed in view of a critical reassessment in the future indications for staging laparotomy concern primarily the extent of disease as provided by present and past experience with surgical staging and, more importantly, by the increasing use of chemotherapy alone or in combination with radiotherapy in the primary management of Hodgkin's disease. First, laparotomy findings prove to be almost invariably negative when clinically the disease is confined either to the upper part of one side of the neck or to the mediastinum (13,16). On the contrary, 60%–80% of the patients with stage III disease were shown to have splenic involvement (13,15,16,19). This was particularly true in those with systemic symptoms and/or with mixed cellularity or lymphocytic depletion. Thus, in both groups of patients the results of laparotomy can be largely anticipated in terms of splenic involvement on the basis of past experience with surgical staging. Furthermore, the radiologic-histologic correlation of retroperitoneal nodes well opacified by contrast material and correctly sampled was proven, in experienced hands, to be very high, ie, at least 90% (10,20,21). The status of high retroperitoneal nodes cannot be properly assessed by a simple random sampling (22). Since radical lymphadenectomy is not advisable for a surgical diagnostic procedure, many radiation therapists add a prophylactic para-aortic field to the upper mantle irradiation even when the results of laparotomy are negative. On the contrary, laparotomy is essential to demonstrate the presence of unsuspected disease in the porta hepatis (19). Positive mesenteric nodes are usually found in $< 5\%$ of untreated patients, and occult findings are practically never observed in CS I and II.

As previously mentioned, the improvement in treatment modalities is probably the most important factor in the reduced need for laparotomy. When Hodgkin's disease is spread above and below the diaphragm and/or systemic symptoms are present, the failure rate after total lymphoid irradiation at 5 years ranges from 25% to 60% despite optimal staging (18,23). The treatment failure is usually due to the reappearance of disease in extranodal areas such as the liver, bone, and marrow (24,25). In particular, in some patients with splenic involvement recurrences usually appear during the first 6 months after radiotherapy (15,26). This is why a number of investigators have either selected intensive combination chemotherapy as the primary treatment for stages IIIA, IIIB, and III$_2$ (12,27) or have started exploring the value of combined radiotherapy plus chemotherapy (25,28–30). Although, at present, combined radiotherapy-chemotherapy should not be regarded as a standard method of treatment, this modality achieved an improved disease-free survival in most studies conducted in patients with intermediate stages, systemic symptoms, or unfavorable histology. If primary chemotherapy used either alone or in optimal sequence with radiotherapy will definitely prove to increase the overall 5-year survival in stages IIB, IIIA, and IIIB as well as in patients with unfavorable histology, then there should be no further indication to use laparotomy, and surgical staging could be limited to lapa-

TABLE 7.—Suggested indications for a more selective use of staging laparotomy after negative liver findings from laparoscopy and negative bone marrow biopsy

Laparotomy can be performed in the presence of—	Laparotomy can be avoided in the presence of—
1. Stage IA (lower cervical, supraclavicular, axillary, inguinal nodes), IIA, and IIIA if primary treatment is radiotherapy alone	1. Stage IA with involvement of upper cervical nodes with unequivocally normal lymphogram
2. Suspicious retroperitoneal nodes on lymphography, when if found histologically positive, primary treatment is radiotherapy alone	2. Stages IA and IIA with disease confined to the mediastinum
3. Splenomegaly if treatment includes subtotal or total nodal irradiation with or without adjuvant chemotherapy; splenectomy avoids excessive irradiation exposure to the left kidney and to the left lower lobe of the lung	3. Stages IB, IIB, IIIB, or IIIA if the treatment program consists of combined radiotherapy-chemotherapy
4. Women asking to preserve their ovarian function during pelvic irradiation	4. Stage III_S on laparoscopy when positive spleen is not associated with splenomegaly and treatment program includes radiotherapy-chemotherapy
	5. Obese, elderly, and emotionally unstable patients as well as patients with other systemic diseases

roscopy plus needle marrow biopsy to confirm or rule out stage IV disease. Table 7 outlines some practical suggestions for a more selective use of staging laparotomy on the basis of the above-mentioned considerations about natural history and treatment options. Furthermore, because of its low morbidity rate laparoscopy can be successfully used to elucidate the patterns of relapse and to verify the completeness of an apparent CR (31).

In conclusion, it can be anticipated that a larger application of laparoscopy combined with needle marrow biopsy and a more widespread use of chemotherapy in groups of patients with an unsatisfactory cure rate after primary radiotherapy will gradually solve the persisting dilemma concerning the routine use of laparotomy. Therefore, the new combined staging procedure and the new concepts of combined modality could eliminate the need for the classic surgical staging in a large fraction of patients. This will eventually decrease the incidence of early and late complications associated with splenectomy in patients receiving intensive treatment, ie, varicella zoster infection and fulminant sepsis (32,33). However, it should be recognized that even without splenectomy there is an increased incidence of varicella zoster in lymphoma patients compared to patients with other malignant disease. Although a death rate of 6.6% has been reported in one particular series (34), the death rate related to staging laparotomy for Hodgkin's disease is estimated to be $< 1\%$, and no deaths occurred in our series of 300 surgically good-risk patients. In our study splenic biopsy was performed only for investigative purposes. Although devoid of major complications, biopsy of the spleen is not recommended as a routine procedure in staging laparoscopy. For patients in whom laparoscopy will substitute for laparotomy both the costs and the hospital stay will be reduced. Laparotomy with splenectomy will probably remain a necessary procedure if the treatment program used is radiotherapy alone in patients with no extranodal disease.

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Noninvasive Monitoring of Adriamycin Cardiotoxicity by "Sphygmo-Recording" of the Pulse Wave Delay (QK_d Interval)[1,2,3]

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SUMMARY

Eighty patients with Ewing's sarcoma, bronchogenic carcinoma, and other neoplasms receiving adriamycin were monitored by a Sphygmo-Recording of the pulse wave delay (QK_d time interval). The QK_d interval, which is the sum of the cardiac pre-ejection period and the pulse transmission time, is sensitive to changes in myocardial contractility and stroke output. The patients were also followed by serial physical examinations, electrocardiograms, chest roentgenograms, serum enzymes, and thyroid function tests; none of these changed during the study period except in a few patients with congestive heart failure (CHF) and/or transient arrhythmias. QK_d showed a significant prolongation (> 30 msec) within 1–3 weeks after adriamycin administration in a high percentage of patients followed closely. The QK_d interval usually returned to pretreatment baseline levels within 4–7 weeks after adriamycin administration. The QK_d often showed repeated and sustained elevations after courses of therapy at 3-week intervals. After adriamycin therapy was discontinued the QK_d usually returned to normal levels. No statistically significant changes in the QK_d were seen in a control group. There were no acute changes in QK_d during adriamycin infusions. Of seven patients receiving cumulative doses of adriamycin > 550 mg/m², three developed CHF. QK_d intervals in all three of these patients had failed to return to the baseline values 2–3 months prior to any other evidence of CHF. This suggests that failure of QK_d to return to pretreatment baseline levels may be of prognostic value. The QK_d interval appears to reflect a high incidence of subclinical adriamycin cardiotoxicity. The technique is simple, noninvasive, rapid, and potentially useful for monitoring patients receiving adriamycin and other potentially cardiotoxic drugs.

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[6]Dr. J. M. Merrill participated in the initial phase of this study. The QK_d measurements were carefully obtained by L. Morris, C. Nunes, R. Huff, B. Baranishyn, D. Johnson, C. Hayworth, and J. Young. R. Proctor and T. Simpkins assisted in data compilation and analysis.

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Adriamycin is an important chemotherapeutic agent which shows significant activity against a variety of neoplasms (1). Adriamycin cardiomyopathy is well documented and currently best avoided by limiting the cumulative dose to 550 mg/m² (2). Approximately 30% of the patients receiving more than this dose will develop congestive heart failure (CHF) (3). A few patients develop CHF at lower doses, particularly when adriamycin is combined with radiation therapy to the chest and/or cyclophosphamide (4,5). Recent data suggest that vigorous therapy for the CHF, particularly if recognized early, can be successful (6). Rinehart et al (7) have found that patients with CHF due to adriamycin cardiotoxicity show alterations in ventricular function measured by systolic time intervals (STI), simi-

lar to the changes seen in patients with CHF of other etiologies. The patients receiving adriamycin therapy also had a progressive dose-dependent prolongation of the cardiac pre-ejection period (PEP), a decrease of the left ventricular ejection time (LVET), and an increased PEP/LVET ratio (7). Rinehart also observed a few patients who had transitory increases of PEP/LVET ratios early in their courses of therapy when the cumulative doses of adriamycin were < 200 mg/m².

Serial noninvasive evaluation of the left ventricular (LV) function may eventually provide a method for prediction of impending cardiac decompensation in time to prevent irreversible CHF. Monitoring might also identify a subpopulation of patients who are at lower risk of developing CHF; these patients might then receive the benefit of higher cumulative doses of adriamycin.

The Sphygmo-Recorder is a device to measure the QK_d interval. QK_d is the time interval between the start of the electrocardiographic (ECG) QRS complex (Q) and the start of the Korotkoff arterial sound (K) over the brachial artery at diastolic pressure (d). The normal range for QK_d is 175–245 msec (mean, 207 msec), with a measurement error of ±7 msec (8). The QK_d and pulse wave contour provide information regarding cardiac function in a variety of normal and disease states (9–12). The QK_d is the sum of the cardiac PEP and the pulse transmission time to the brachial artery. In a coronary care unit, the QK_d and PEP showed a correlation of $r = 0.95$ (13). Thus the QK_d interval, like the PEP, is a dynamic measurement useful as an index of cardiac contractility.

In the present study, we have utilized the QK_d interval for monitoring patients receiving adriamycin in various treatment regimens.

METHODS AND MATERIALS

The QK_d interval may be measured on a high-speed two-channel strip chart recorder (100 or 200 mm/sec) displaying ECG lead I or II on one channel and the arterial blood pressure sounds on the other (14), as the blood pressure cuff is deflated from above systolic to below diastolic pressure. A plot of the QK_d intervals versus cuff pressure provides a calibrated contour of the arterial pressure wave. In the present study, by use of suitable electronic instrumentation (Sphygmo-Recorder) (15,16), the pulse wave contour and QK_d interval were displayed automatically on the face of a storage oscilloscope and then photographed by a Polaroid camera. The means of triplicate measurements were used. Measurements were obtained for most patients at weekly or biweekly intervals. The maximum change in the QK_d interval above pretreatment baseline after each course of chemotherapy was noted for each patient. Thus, each patient also served as his own control. The same apparatus was used to record heart sounds.

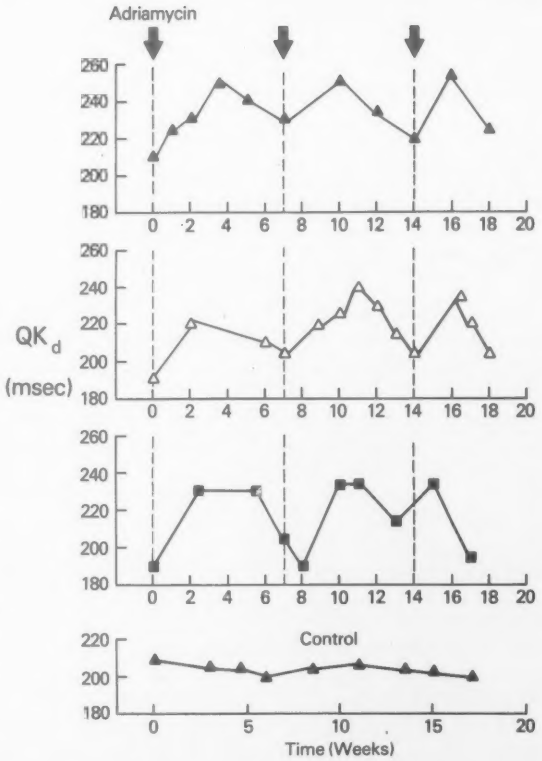

FIGURE 1.—Comparative serial QK_d intervals in 3 patients with Ewing's sarcoma receiving sequential adriamycin therapy alternating with cyclophosphamide and vincristine (top) and 1 representative control subject not receiving adriamycin (bottom).

Serial QK_d measurements were obtained on 11 normal subjects (group 1). Serial measurements were also obtained for the QK_d interval in 32 young adult patients with Ewing's sarcoma who were receiving adriamycin (75 mg/m² iv every 7 weeks) in sequence with cyclophosphamide (40 mg/kg iv) and vincristine (1.4 mg/m² iv)[7] as an adjuvant to radiation therapy (17). In 22 of these patients (group 2), QK_d measurements were usually obtained at 1- or 2-week intervals, and occasionally every 3 weeks. In ten of these patients with Ewing's sarcoma, it was impossible to obtain QK_d measurements as frequently (at least once per month) (group 3), and the

[7]Cyclophosphamide: NSC-26271; CAS reg. No. 6055-19-2; 2*H*-1,3,2-oxazaphosphorine, 2-[bis(2-chloroethyl)amino]tetrahydro-, 2-oxide, monohydrate.

Vincristine: NSC-67574; CAS reg. No. 2068-78-2; leurocristine, sulfate (1:1).

values were obtained at 4–7-week intervals. Most of the patients with Ewing's sarcoma were receiving adriamycin for the first time. However, some had received adriamycin > 3 months previously but with cumulative doses < 300 mg/m².

Serial QK_d measurements were also obtained in 12 patients with bronchogenic carcinoma (group 4) receiving radiation therapy to the pulmonary lesion(s) (3000 rads over 3 weeks) and intermittent cycles of combination chemotherapy with adriamycin (40 mg/m² iv), cyclophosphamide (900–1500 mg/m² iv), and vincristine (1.4 mg/m² iv) every 3 weeks. Five patients with other neoplasms receiving adriamycin were also followed (group 5). Chest roentgenograms, ECGs, and laboratory tests including cbc counts and serum enzymes were obtained serially on all patients. Serial thyroid function tests were obtained in five patients.

RESULTS

Table 1 summarizes the results. The mean and standard deviation, the number of patients, the standard error of the mean, and the range of observations are shown in each category.

Controls

In 11 normal subjects (group 1) and in 12 patients with Ewing's sarcoma receiving chemotherapy other than adriamycin (data not shown), frequent serial measurements rarely showed changes in QK_d intervals > 30 msec. The mean of triplicate values for any session (day) had a standard error of 4.2 msec. However, the between-day variability for any one subject was 14 msec. The between-subject variability was ±24 msec. A representative case is shown in figure 1 (bottom panel). Accordingly, we regarded a change in QK_d of ≥ 30 msec as a "significant" change; such a change would be significant at the $P < 0.05$ level, representing a change of slightly more than two standard deviations of measurement error. (The exact P value may differ from this since we analyzed the maximum change in the QK_d, among several measurements.)

Single-Infusion Studies

Four patients were studied during adriamycin infusions. The QK_d, heart rate, blood pressure, and ECG were monitored continuously during a 15-minute infusion and immediately after infusion for a

TABLE 1.—Summary of QK_d intervals and changes following repeated cycles of adriamycin therapy*

Group No.	Baseline QK_d intervals		Maximum change in QK_d during 1st course		QK_d on 1st day of 2nd course		Maximum change in QK_d during 2nd course		QK_d on 1st day of 3rd course		Maximum change in QK_d during 3rd course	
1. Control (3 readings/course)	205.6	24.2	12.8	21.7	9.0	23.4	2.9	29.4	4.9	16.7	13.7	10.7
	11	7.3	11	6.6	11	7.1	9	9.8	7	6.2	6	4.3
	165	254	−17	54	−35	36	−56	50	−22	23	−2	27
2. Ewing's sarcoma (frequent followup)	199.6	26.6	24.0	23.1	14.8	20.6	21.8	19.4	13.7	18.0	30.5	18.7
	22	5.7	15	6.0	12	5.9	15	5.0	12	5.2	11	5.6
	153	255	−8	83	−24	56	−18	48	−20	37	−4	53
3. Ewing's sarcoma (infrequent followup)	190.9	35.4	2.1	6.7	9.5	17.0	7.0	28.4	8.2	18.2	9.1	13.7
	10	11.2	8	2.4	6	6.8	4	14.2	5	8.1	8	4.8
	132	250	−11	11	−7	35	−29	33	−9	38	−13	30
2 and 3 combined—Ewing's sarcoma (frequent and infrequent followup)	196.8	29.3	16.4	47.6	13.1	19.1	18.7	21.6	12.1	17.7	21.5	19.7
	32	5.2	23	9.9	18	4.5	19	5.0	17	4.3	19	4.5
	132	255	−11	83	−24	56	−29	48	−20	38	−13	53
4. Bronchogenic carcinoma	191.5	22.1	13.3	22.1	17.0	14.7	31.3	28.3	33.1**	34.8	26.4	31.0
	19	5.1	11	6.7	8	5.2	12	8.2	13	9.7	10	9.8
	142	230	−33	48	0	36	−14	93	−27	95	−22	74
5. Other	210.6	24.8	24.7	24.8	22.3	26.1	24.5	22.1				
	5	11.1	3	14.3	3	15.1	4	11.1				
	183	240	−3	45	0	51	0	49				

*Within each category: top line = mean and standard deviation; second line = No. of patients and standard error of the mean; third line = range of observations.

period of 1 hour. No changes were seen over this period of time. The first change was a 10-msec increase in QK_d on Day 4 in two patients, which became significant (25 and 50 msec) by Day 8. Subsequent data on the other two patients were not obtained for 2 weeks, at which time both showed significant prolongation of QK_d (30 and 35 msec).

Longitudinal Studies

In 22 patients with Ewing's sarcoma (group 2), the mean maximal prolongation of the QK_d interval after the first course of adriamycin was 24.0 msec, with a standard error of 6.0 and a range of −8 to 83 msec (table 1). About the same mean change (21.8 msec) relative to initial baseline QK_d was seen in 15 of these patients after a second course of adriamycin and in 11 patients after a third. In most of these patients the QK_d returned to baseline prior to the next scheduled course of adriamycin therapy 7 weeks later (table 1).

In ten patients with sarcoma on the same treatment protocol who were followed infrequently (less than once per month) (group 3), the frequency of a change of > 30 msec was lower than in group 2. Combining the results from groups 2 and 3 about half of the patients had prolongation of the QK_d interval of > 30 msec. The percentage of patients with this change did not appear to increase between the first and third course of treatment.

In most patients with bronchogenic carcinoma (group 4), there was a significant increase in the QK_d after the second course of therapy. The mean prolongation of the QK_d after the second and third cycles of therapy was increased relative to the change in the first course. The change in the mean QK_d was comparable to that seen in patients with Ewing's sarcoma receiving adriamycin every 7 weeks. In the patients with bronchogenic carcinoma receiving adriamycin every 3 weeks, the QK_d generally failed to return to baseline and remained significantly prolonged at the time of administration of the second and third cycles of chemotherapy. Results for three representative patients are shown in figure 2.

Seven of the patients studied received a total adriamycin dose of > 550 mg/m². Each of these patients had previously been treated with other drugs without continued benefit, and their disease appeared responsive to additional adriamycin. The decision to use these high doses was made by physicians not involved in the present study. Three of these patients developed overt CHF.

In a 35-year-old white woman receiving adriamycin therapy for metastatic breast carcinoma, repeated elevations of QK_d were observed which did not return to within 20 msec of baseline levels a few months prior to overt CHF (fig 3). After a cumulative dose of 950 mg/m² she developed CHF with tachycardia, gallop, cardiomegaly, hepatomegaly, peripheral edema, effusions, and evanescent arrhythmias. Note the gradual increase of the QK_d intervals in this patient prior to the onset of CHF.

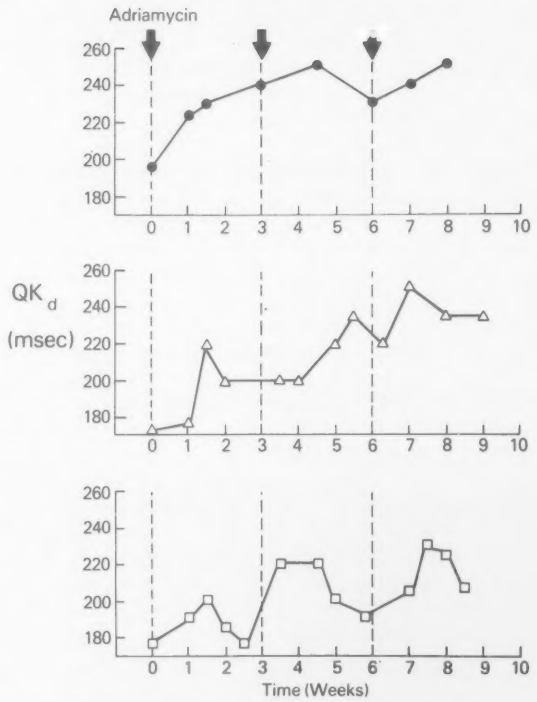

FIGURE 2.—Serial QK_d intervals in 3 patients with bronchogenic carcinoma plotted in relation to chemotherapy.

The QK_d values obtained when the patient initially developed CHF were quite erratic. The QK_d was markedly prolonged (to 250 msec) when the patient was acutely decompensated, and decreased toward normal with clinical improvement after hospitalization and intensive therapy with dopamine infusion and diuretics. Ventricular (S3) and atrial (S4) gallops were detected by the recording of heart sounds over the apex with the Sphygmo-Recorder (16) (fig 4). By automatically aligning the sounds from several beats with respect to the QRS complex, one can detect faint or intermittent third and fourth heart sounds which might otherwise escape detection. The second patient to develop CHF was a 22-year-old man with metastatic Ewing's sarcoma receiving cycles of adriamycin for the second time. After a dose of 600 mg/m² he developed severe CHF which did not respond to vigorous therapy with

digitalis and diuretics and he died. The QK_d values prior to development of CHF, measured every 4–7 weeks, also revealed a failure to return to near baseline values on the days of adriamycin infusion. Photomicrographs of the myocardium showed marked fragmentation and edema consistent with adriamycin cardiomyopathy, and electromicroscopy showed disruption of mitochondria and myolysis. The third patient is a 48-year-old woman with metastatic breast carcinoma. Her QK_d failed to return to near baseline after a dose of 600 mg/m² of adriamycin given every 4 weeks. She developed orthopnea, dyspnea on exertion, and a ventricular gallop, and the adriamycin has been stopped. A fourth patient showed significant elevation of baseline QK_d, with no other evidence of CHF, at the time of her two last scheduled treatments (after a total dose of 680 mg/m²) and adriamycin was discontinued. Within 2 months the QK_d has returned to baseline and there was no evidence of CHF. In the three remaining patients in this series (total doses of 675, 605, and 600 mg/m²), the QK_d has consistently returned to near baseline similar to group 3, and there is no evidence of CHF.

With the exception of the three patients who developed CHF, there were no significant changes in the physical examination of the heart, pulse rate, chest roentgenogram, or serum enzymes (SGOT, CPK, LDH) coincident with the prolongation of the QK_d. Two patients developed premature atrial beats and one developed frequent ventricular premature beats with bigeminy, but no other ECG abnormalities were noted. Thyroid function studies (serum thyroxine, thyroid-stimulating hormone, thyroid-binding globulin, and triiodothyronine) did not change in five patients at the time of prolongation of the QK_d interval. Decreases in hemoglobin, usually associated with the shortened QK_d, were seen consistently 2–3 weeks after chemotherapy at a time when the QK_d was usually elevated.

DISCUSSION

These observations reveal a transitory impairment of cardiovascular function appearing 1–3 weeks after a single dose of adriamycin in a great proportion of patients, as evidenced by a prolonged QK_d interval. The QK_d interval usually returned to pretreatment baseline within 4–7 weeks. The overall incidence of significant prolongation of the QK_d may be even higher because the ten patients with Ewing's sarcoma in whom we failed to observe significant changes in QK_d were followed less frequently. The experience with this group (group 3) is compatible with that of group 2, in which most patients showed a return of the QK_d to the baseline by the time of administration of the next course of therapy. These results were also compatible with those of Burg et al (18) who failed to find changes in STIs in patients followed less frequently and apparently at random times with respect to administration of adriamycin. The present results suggest that long intervals between measurements may have resulted in the negative finding.

In group 4, the QK_d usually did not return to

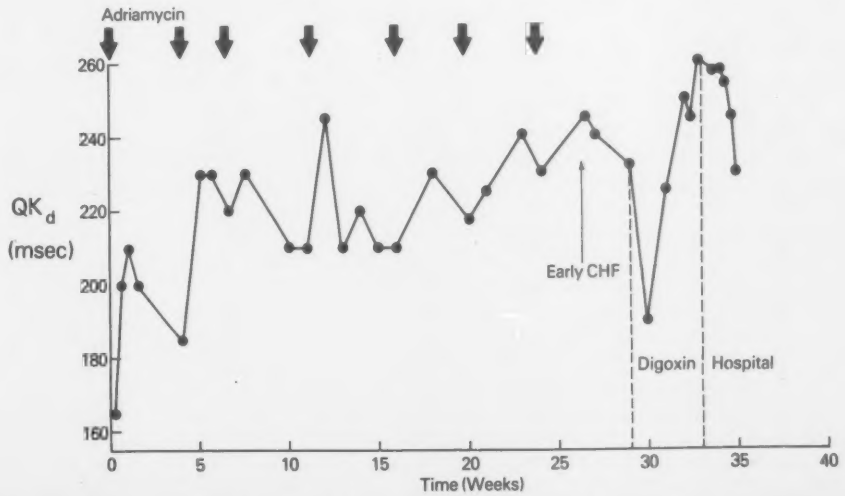

FIGURE 3.—Serial QK_d measurements in a patient with metastatic breast carcinoma who developed CHF after a cumulative dose of 960 mg/m² of adriamycin. At the point indicated the patient was hospitalized and treated with diuretics and iv dopamine. As her cardiac status improved, the QK_d returned to the baseline values.

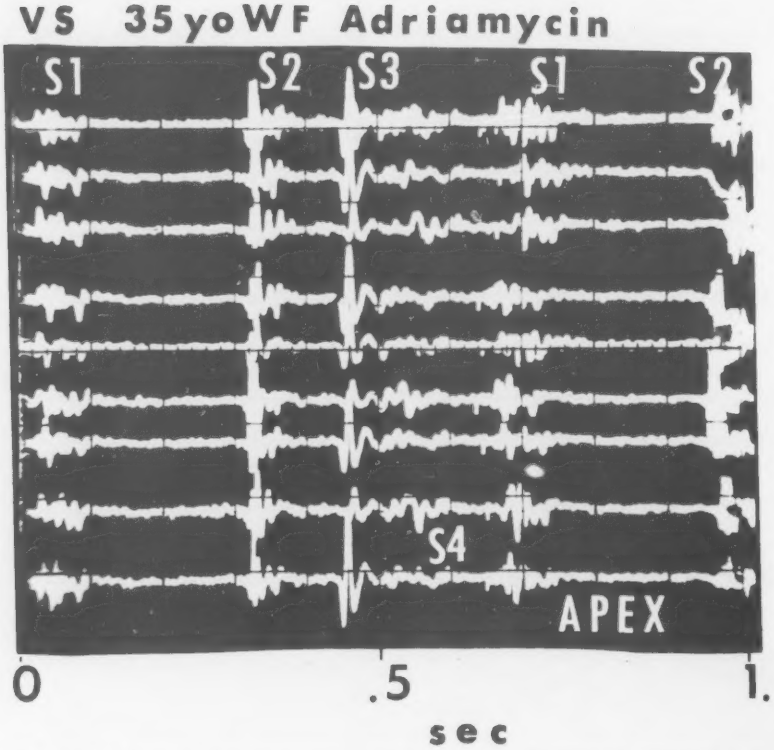

FIGURE 4.—Heart sounds recorded at the apex in the patient studied in fig 3 using the Sphygmo-Recorder (16) with a 30–1000-Hz band pass filter.

baseline levels by the time (3 weeks) subsequent cycles were administered. The more frequent treatment, or the combination with cyclophosphamide and mediastinal radiation, may have been responsible for the failure of the QK_d to return to baseline. Age may also be an important factor because the group with bronchogenic carcinoma were older adults and the group with Ewing's sarcoma were young adults and children.

The present results confirm the finding of transient alteration of STIs (7) in patients receiving adriamycin. They are also consistent with echocardiographic studies which disclosed decreased cardiac ejection fractions in patients receiving total doses of adriamycin > 400 mg/m² (19). The QK_d interval is a sensitive, simple, reproducible measurement readily obtained by a technician or nurse with minimal instruction in a few minutes at the clinic or bedside. In contrast to the use of standard STIs, the QK_d measurement does not require recording of the heart sounds (with the need for patient to disrobe), carotid pulse wave contours, or any calculations. Measurement of the QK_d interval in patients receiving adriamycin is a potentially useful technique. A direct comparison with standard STIs in the same patient would be desirable to determine the relative effectiveness of the two methods. However, this was not feasible in the present study.

The recurrent transitory increases in QK_d, occurring 1–3 weeks after adriamycin therapy, probably represent a direct toxic effect on the myocardium. It is conceivable, though unlikely, that there could be an effect mediated through the autonomic nervous system. The absence of significant changes in heart rate reduces the likelihood of this possibility. Likewise, depression of thyroid function is an unlikely explanation for the observed changes in QK_d; there was no clinical or chemical evidence for hypothyroidism.

Recent studies have shown a depression of DNA synthesis in the myocardium of mice occurring 6–8 days after administration of adriamycin (20). This effect was dose dependent and temporally related to fatal cardiotoxicity. An autopsy of patients receiving adriamycin who died of noncardiac causes demonstrated a high incidence of the histologic changes

consistent with adriamycin cardiomyopathy, although of a lesser degree, in those who had received low cumulative doses of adriamycin (6). It is possible that the changes in QK_d are indicative of such clinically occult myocardial damage.

Transient ECG abnormalities have been reported to occur in 6%–30% of the patients treated with adriamycin, usually within the first few days after administration (3,21,22). Transitory arrhythmias were observed in three of our patients, but we did not observe major conduction defects, such as left bundle branch block. Thus, it does not appear that conduction defects were responsible for the prolongation of the QK_d interval. Consistent with the findings of others (7,18) there were no other signs of myocardial cellular damage (ie, ECG or serum enzyme changes) in any of our patients (except two with overt CHF). It is conceivable that sensitive radioimmunoassays for other markers, such as myoglobin, actin, and/or myosin, might reveal such changes.

In patients with neoplasms receiving chemotherapy, one might expect greater variability in QK_d than in normal subjects. This variability may be due to anemia, fever, pain (and adrenergic response), increased metabolism, and/or arterio-venous shunting through tumor beds. This variability is only partially eliminated by use of the initial QK_d value as the baseline for each subject, ie, using each subject as his own control. A control group with Ewing's sarcoma not receiving adriamycin and normal controls (group 1) showed no significant QK_d changes.

The results in the seven patients who received cumulative doses of adriamycin > 550 mg/m^2 are provocative: they suggest that in patients receiving high-dose adriamycin, failure of QK_d to return to within 30 msec of baseline values might be associated with increased risk of subsequent development of overt CHF. However, whether changes in QK_d or similar noninvasive measures of cardiac function can be used to accurately identify populations relatively susceptible to adriamycin cardiomyopathy remains to be answered. This would require a much larger patient population and a longer followup than is available to date. The present study also suggests that frequent (weekly) and intensive history-taking and frequent phonocardiograms to detect third heart sounds will assist in early detection of cardiotoxicity. In view of the present recommended dose limitation of 550 mg/m^2, the number of patients developing CHF after receiving adriamycin therapy should (hopefully) be quite small. This would make it difficult to establish whether transitory decreases in QK_d are predictive of increased risk of incipient or impending CHF. Such information may be obtainable in the clinical trials of analogs of adriamycin for which the cardiotoxic threshold is unknown.

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Phase II Studies of Dianhydrogalactitol and VP-16-213 in Colorectal Cancer[1,2,3]

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SUMMARY

Phase II chemotherapy trials of dianhydrogalactitol and VP-16-213 were conducted in patients with metastatic colorectal cancer who had measurable malignant disease which served as indicators of response to therapy. Dianhydrogalactitol was given in a 5-day course at a dosage of 30 mg/m²/day. Toxic reactions included nausea, vomiting, leukopenia, thrombocytopenia, and anemia. There was a definite tendency to a compounding of hematologic toxicity with repeated courses. No evidence of objective therapeutic response was observed among 30 patients treated. VP-16-213 was given at a dosage of 130 mg/m² on Days 1, 3, and 5. Toxic reactions included nausea, vomiting, alopecia, leukopenia, thrombocytopenia, and anemia. Hematologic toxicity was more severe in patients with elevated serum bilirubin levels. No evidence of objective therapeutic response was observed among 28 patients treated.

[Cancer Treat Rep 60:1247–1250, 1976]

PHASE II STUDY OF DIANHYDROGALACTITOL

The dihalohexitols, dibromodulcitol and dibromomannitol, are cytotoxic agents that inhibit the growth of a number of experimental animal tumors and produce characteristic effects on blood elements (1). These compounds have previously been regarded as alkylating agents because of their chemical reactivity and cross resistance to alkylating agents, but their mechanism of action is too complex to be explained by alkylation alone.[6] The major activity of these halogenated hexitols is thought to be related to their conversion to the diepoxide, dianhydrogalactitol.

Animal studies utilizing dianhydrogalactitol found leukopenia and anemia to be the major toxic effects. Few clinical trials are complete, but our studies revealed that a dose of 30 mg/m² administered iv daily for 5 days produced definite but clinically tolerable toxicity (2).

Because dianhydrogalactitol has not been evaluated in colorectal carcinoma (a major cancer problem), this phase II trial was established to look for therapeutic activity by administering the drug in the above schedule.

Materials and Methods

Patient Selection

Thirty patients with histologically confirmed adenocarcinoma of the colon and rectum were selected for this study. Each patient was ambulatory and was maintaining an adequate nutritional state before treatment. We required a measurable lesion to serve as an objective indicator of response. Malignant hepatomegaly was acceptable if hepatic metastasis was biopsy proven and if on quiet inspiration the liver measured at least 5 cm below the xiphoid or costal margins. Liver scans were acceptable if

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[2]Supported by contract N01-CM-02066 from the Division of Cancer Treatment (DCT), National Cancer Institute (NCI), National Institutes of Health, Department of Health, Education, and Welfare.

[3]Dianhydrogalactitol: NSC-132313; CAS reg. No. 23261-20-3; 1,2:5,6-dianhydrogalactitol.

VP-16-213: NSC-141540; 4'-demethylepipodophyllotoxin 9-(4,6-*O*-ethylidene-β-D-glucopyranoside).

Both drugs were supplied by the Cancer Therapy Evaluation Program, DCT, NCI.

[4]Department of Medicine, University of Missouri Medical Center, Columbia.

[5]Mayo Clinic and Mayo Foundation, Rochester, Minn.

[6]Handelsman H. Clinical brochure: galactitol, 1,2:5,6-dianhydro (NSC 132313). Prepared by the DCT, NCI, Bethesda, Md.

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there was a clearly defined perfusion defect measuring at least 5 cm in the longest diameter.

Therapy was delayed for at least 1 month after any prior radiation or chemotherapy, 3 weeks after any abdominal surgical procedure, and 4 weeks after surgery involving resection or anastamosis. Leukocyte counts of < 4000 cells/mm^3 or platelet counts of < 130,000 platelets/mm^3 were contraindications to participation in the study.

General Treatment Methodology

Before treatment the following laboratory studies were performed: hemoglobin level; leukocyte, platelet, and differential counts; serum creatinine, bilirubin, alkaline phosphatase, SGOT, calcium, carcinoembryonic antigen, and immunoglobulin levels; and chest roentgenogram. After treatment, leukocyte counts were obtained twice weekly and platelet counts once weekly. At 4 and 8 weeks a physical examination was performed, indicator lesions were remeasured, and all pretreatment laboratory studies were repeated. Those patients with objectively improved or stable disease continued to receive treatment. Progressive disease was the indication for cessation of therapy.

Our criteria for an objective response were: a reduction by at least 50% of the product of the longest perpendicular diameters of the most clearly measurable lesion; a reduction by at least 30% of the sum of the liver measurements below each costal margin and xiphoid process (if malignant hepatomegaly was the indicator); no increase in any other lesion and no new area of malignant disease; and no significant deterioration in weight, symptoms, or performance status.

Drug Administration

Dianhydrogalactitol was dissolved in sodium chloride for injection and administered as a rapid iv push at a dose of 30 mg/m^2 on each of 5 consecutive days. Phenothiazine antiemetics were administered as needed. A second course was given on Days 29–33 and any subsequent courses were administered every 5 weeks with a dose of dianhydrogalactitol adjusted to produce definite but clinically tolerable toxicity. Initiation of subsequent courses was deferred until leukocyte and platelet counts had returned to normal levels.

Results

Of our 30 patients, 17 had had prior chemotherapy. All had had 5-fluorouracil and 13 of 17 had received other agents in addition, usually methyl-CCNU, *cis*-dichlorodiammineplatinum(II), or ICRF-159. Two patients had received radiotherapy.

Toxicity

Toxic reactions after therapy are shown in table 1. The rate of occurrence and severity of toxic reactions were essentially the same between patients with previous exposure to chemotherapy and patients without prior treatment. Nausea and vomiting were frequent but were usually well controlled with phenothiazines. Hematologic toxicity was more common with one third of the patients experiencing leukopenia of < 2000 cells/mm^3 and one fifth experiencing thrombocytopenia of < 50,000 platelets/mm^3. The median day of nadir was 17 days for platelets and 21 days for leukocytes; recovery was occasionally delayed. Of the ten patients who received more than one course of therapy, four had their treatment postponed (up to 14 days) because of persistent leukopenia or thrombocytopenia. There was definite evidence of a compounding of hematologic toxic effects in some patients. Of six patients re-treated with the same dose, three showed no change in the degree of leukopenia or thrombocytopenia. One patient showed mild leukopenia with the second course (3700 cells/mm^3) after showing none with the first course. One patient who had had no hematologic depression with the first course had leukopenia to a nadir of 2400 cells/mm^3 which persisted for 39 days with the second course. A third patient had a low wbc count of 3500 cells/mm^3 and a low platelet count of 82,000 platelets/mm^3 with the first course and nadirs of 1300 cells/mm^3 and 15,000 platelets/mm^3 respectively with the second course.

Therapeutic Results

Of the 30 patients treated with a 5-day course of dianhydrogalactitol, 20 showed objective evidence of progression at 4 weeks and were not re-treated. Six patients who had stable disease at 4 weeks had progressive disease by 8 weeks after two courses of

TABLE 1.—Toxic reactions of 30 patients treated with dianhydrogalactitol

Toxic reaction	No. of patients (%)
Nausea	15 (50)
Vomiting	12 (40)
Leukopenia	
2000–4000 cells/mm^3	13 (43)
< 2000 cells/mm^3	10 (33)
Thrombocytopenia	
50,000–100,000 platelets/mm^3	7 (23)
< 50,000 platelets/mm^3	6 (20)
Anemia (decrease in hemoglobin > 2 g/100 ml)	12 (40)

therapy, and four patients had stable disease and then progressive disease after three courses. In three patients disease progression was evidenced by the appearance of new lesions and in the remaining 27 patients by progression of known lesions. Only two patients reported an increase in performance status, one in the face of progressive disease. No patient showed evidence of objective improvement.

Discussion

In this phase II study of dianhydrogalactitol administered iv for 5 days, the primary toxic effects were nausea and vomiting, with later leukopenia and thrombocytopenia. Hematologic toxicity was the major dose-limiting factor. There was evidence of a compounding of hematologic toxicity with repeated treatment. No evidence of beneficial effect was seen in the 30 patients we treated, therefore we must conclude that dianhydrogalactitol in this dose schedule is ineffective for colorectal cancer. These results are perhaps not surprising since they parallel the essentially negative experience reported with dibromodulcitol in carcinoma of the colon and rectum (3).

PHASE II STUDY OF VP-16-213 (VP-16)

VP-16, a semi-synthetic product derived from podophyllotoxin, has been shown to have appreciable antitumor effect in several animal tumor models.[7] It has a cytostatic action in vitro which prevents cells from entering mitosis or destroys them in the premitotic phase (4). It has been shown to be oncostatic in vivo in trials with L1210 leukemia, P815 mastocytoma, and P1534 leukemia (4).

VP-16 has not had a phase II evaluation in large bowel carcinoma although Falkson and Falkson (5) described two of eight patients treated with oral VP-16 as "improved," and the European Organization for Research on the Treatment of Cancer found no effect in three patients (6). We have used a dose schedule of 130 mg/m² iv on Days 1, 3, and 5 repeated every 4 weeks in phase I trials with nausea, vomiting, alopecia, and leukopenia as the toxic effects (7).

The objective of this study was to assess the activity of VP-16 in maximally tolerated clinical dosage against advanced colorectal carcinoma. We utilized two groups of patients, one previously treated with other chemotherapy agents and one previously unexposed to any such agents.

[7]Goldsmith MA. Clinical brochure: 4'-dimethyl-epipodophyllotoxin-β-D-ethylideneglucoside (NSC 141540) VP-16-213. Prepared by the DCT, NCI, Bethesda, Md, Jan 1973.

Materials and Methods

Patient selection and general treatment methodology were the same as those described for the dianhydrogalactitol study.

Drug Administration

VP-16 was supplied in 5-ml ampules, each containing 100 mg of active compound. After dilution in sodium chloride, the drug was administered iv at a dose of 130 mg/m² on Days 1, 3, and 5. A second course was given on Days 29, 31, and 33. Any subsequent courses were given at 5-week intervals. Doses of VP-16 were adjusted in subsequent courses to produce definite but clinically tolerable toxicity.

Results

A total of 28 patients were treated with VP-16, 11 men and 17 women. The mean age was 60 years with a range of 29–79 years. Fifteen patients had had prior chemotherapy. All previously treated patients had received 5-fluorouracil. It had been combined with either radiotherapy or other chemotherapeutic agents in 14 of the 15 patients. Eight of the 15 patients had been exposed to methyl-CCNU. Four patients had had prior radiation therapy.

Toxicity

Table 2 lists the toxic reactions to VP-16 treatment experienced by our patients. Nausea and vomiting were common on the day of treatment but were not severe enough to alter drug administration. Alopecia was nearly universal in those patients who were evaluable for this toxic effect. Bone marrow depression was the major limiting toxic effect. Leukopenia of moderate degree (< 4000 cells/mm³) was common (18 of 28 or 68% of the patients) and

TABLE 2.—Toxic reactions of 28 patients treated with VP-16

Toxic reaction	No. of patients (%)
Alopecia	20 (71)
Nausea	15 (64)
Vomiting	10 (35)
Leukopenia	
> 2000–< 4000 cells/mm³	19 (68)
< 2000 cells/mm³	3 (11)
Thrombocytopenia	
> 50,000–< 100,000 platelets/mm³	9 (14)
< 50,000 platelets/mm³	1 (4)
Anemia (decrease in hemoglobin > 2 g/100 ml)	7 (25)
Drug-related death	1 (4)

TABLE 3.—VP-16-induced leukopenia and serum bilirubin levels

Nadir of leukopenia (cells/mm³)	No. of patients with serum bilirubin (mg/100 ml)—		
	< 1.5	> 1.5–5	> 5
> 4000	5	—	1
< 4000–> 2000	16	3	—
< 2000–> 1000	1	—	—
< 1000	—	—	2
Total	22	3	3

occasionally severe (< 2000 cells/mm³ in three of 28 or 11% of the patients). Thrombocytopenia was less severe (table 2). The nadir of leukocyte and platelet counts usually occurred 10–14 days after treatment.

There seemed to be a correlation between impairment of liver function as expressed by elevation of serum bilirubin and the degree of toxicity as expressed by leukopenia (table 3). Among those patients with normal or only moderately elevated bilirubin, toxicity was tolerable and quite reproducible. The lowest leukocyte count in this group was 1800 cells/mm³. Among three more deeply jaundiced patients, however, two had profound leukopenia (< 1000 cells/mm³). One of these patients died from hepatic failure 11 days after treatment with drug toxicity presumably contributing to death. We could not clearly relate drug toxicity to abnormal levels of SGOT or alkaline phosphatase.

Therapeutic Results

Of the 28 patients selected for treatment, there was one early death (hepatic failure). Thirteen patients showed objective progression at 4 weeks and were not re-treated. An additional 11 patients had stable disease at 4 weeks but had progressive disease at 8 weeks. Two patients had stable disease for 13 weeks before progression and one patient for 18 weeks. One patient has continued with objectively stable disease for 23 weeks and has had improvement in performance status. No patient has had any evidence of objective improvement.

Discussion

In this phase II study, VP-16 was administered on Days 1, 3, and 5 with leukopenia, thrombocytopenia, alopecia, and nausea and vomiting as the major toxic effects. The dose-limiting toxicity was hematologic with one death related to pancytopenia in a severely jaundiced patient. We would recommend caution in the use of this drug for patients with jaundice, particularly since the drug is partially excreted through the bile.[8] Overall toxicity in non-jaundiced patients was moderate and within clinically tolerable limits.

No evidence of therapeutic activity was seen among our 28 patients with large bowel carcinoma and we conclude that VP-16 using this program has no beneficial effect.

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[8] *See* footnote 7.

Phase I Alternate-Day Dose Study of Chromomycin A_3[1,2]

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SUMMARY

Chromomycin A_3 was given to 43 patients with metastatic cancer in order to determine the tolerable dose when the drug was administered on an every-other-day dose schedule for a total of five iv push injections, with the course of therapy being repeated every 4 weeks. At least three patients were entered at each dose level, graduated in 0.1-mg/m² increments between 0.7 and 1.6 mg/m². The most common (19 patients) side effect was nausea and/or vomiting, but this was usually mild, lasted for a few hours, and diminished in severity with repeated injections. Skin necrosis due to drug extravasation was a problem early in the study, but was eliminated by injecting the drug through iv tubing. Transient elevations in SGOT and alkaline phosphatase levels were observed, but proved not to be of serious consequence. Renal toxicity proved to be the limiting factor in therapy. However, a dose level of 1.3 mg/m² was found to be a tolerable level of drug administration in previously untreated patients. Objective tumor responses were noted in four patients (Hodgkin's disease, embryonal rhabdomyosarcoma, adenocarcinoma of the lung, and malignant melanoma).

[Cancer Treat Rep 60:1251–1255, 1976]

Chromomycin A_3,[5] an antibiotic isolated from *Streptomyces griseus* No. 7, is structurally related to mithramycin, differing only in the pentose structure (1–3). The action of chromomycin A_3 is believed due to the inhibition of DNA-dependent RNA polymerase and of DNA polymerase.

Published reports from Japan and South Africa have suggested that chromomycin A_3 is extremely effective in a wide variety of tumors (4–6). Responses have been reported in malignant lymphomas (33 of 45 patients; 73%), uterine and cervical carcinomas (11 of 25 patients; 44%), ovarian carcinomas (17 of 38 patients; 45%), bronchogenic carcinomas (26 of 64 patients; 41%), hepatomas (seven of 20 patients; 35%), stomach cancers (55 of 156 patients; 35%), colorectal cancers (11 of 32 patients; 34%), breast carcinomas (three of 26 patients; 11%), sarcomas (five of 11 patients; 45%), trophoblastic carcinomas (eight of 12 patients; 67%), and head and neck cancers (nine of 14 patients; 64%).

Studies performed in the US have reported nausea and vomiting, hypocalcemia, reduced calcium clearance, increase in BUN, increase in serum creatinine, and decrease in creatinine clearance (7,8), and have resulted in a general discontent for the use of this agent. Daily doses of $\geq$ 0.9 mg/m² have produced almost uniform impairment of renal function. Slow iv infusion did not negate the renal toxicity found to be acute tubular necrosis by renal biopsy (7).

[1]Received May 7, 1976; revised Aug 4, 1976; accepted Aug 19, 1976.

[2]Chromomycin A_3: NSC-58514; CAS reg. No. 7059-24-7. Supplied by the Drug Research and Development Program, Division of Cancer Treatment, National Cancer Institute (NCI).

[3]This study was performed by the Western Cancer Study Group (WCSG) (Joseph R. Bateman, chairman). The following investigators participated in this study: R. D. Reynolds, J. I. Fisher, and P. A. Jensen (David Grant US Air Force [USAF] Medical Center, Travis AF Base [AFB], Calif); T. F. Pajak (presently with Acute Leukemia Group B) and J. R. Bateman (University of Southern California, Los Angeles). Other WCSG participants are: J. Lewis and B. Greenberg (University of California, Davis); M. Corder (University of Iowa); E. Flannery (Letterman General Hospital); C. Kardinal (Ellis Fischel State Cancer Hospital); F. Bull (University of Michigan); and L. Irwin and S. Nystrom (University of Southern California).

[4]We wish to thank Millie Pricer and Alethea McClain for their technical assistance.

[5]Slavik M, and Carter SK. Clinical brochure: chromomycin A_3 (NSC-58514) (Toyomycin). Prepared by the NCI, Bethesda, Md, 1972, 39 pp.

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Because mithramycin therapy has been associated with RNA synthesis recovery in the liver within 72 hours, a phase I study of chromomycin A_3 was designed to test the possibility of using an alternate-day treatment program in order to minimize the known toxic effects.

MATERIALS AND METHODS: PATIENTS

All patients had histologically proven malignant disease without reasonable expectation of better response to other chemotherapy agents, radiotherapy, or surgery. A Karnofsky performance score of $\geq$ 50 (9) and a life expectancy of $\geq$ 90 days were required. Required intervals from previous therapy were 4 weeks for chemotherapy or radiotherapy and 3 weeks for surgical procedures. Those with abnormal renal or hepatic function were considered ineligible. Hematologic criteria for entry in the study included a wbc count > 4000 cells/mm^3, platelet count > 150,000 cells/mm^3, hematocrit level > 28 vol%, hemoglobin level > 9.5 g/100 ml, and bone marrow cellularity > 30%. Other pre-treatment study requirements included measurement of differential wbc and reticulocyte counts, serum electrolytes (sodium, potassium, chloride, carbon dioxide, calcium, and phosphorus), chest roentgenograms, serum protein electrophoresis, bilirubin, alkaline phosphatase, CPK, LDH, urinalysis, electrocardiogram (ECG), and a signed informed consent.

Chromomycin A_3 was administered by iv push on Days 1, 3, 5, 8, and 10. The 0.5-mg vials were reconstituted with 10 ml of sterile water and the unused partial vials were discarded. Repeat courses were given every 29 days. The starting dose was 0.7 mg/m^2. It was planned to increase the dose by 0.1 mg/m^2 in increments of three patients. The dose was kept constant in each patient.

The cbc count, BUN, creatinine, urinalysis, calcium, and phosphorus were repeated on Days 5, 8, 15, 22, and 29. Creatinine clearance, liver function, and serum electrolytes were repeated on Days 8, 15, 22, and 29. Values for the cbc and BUN were required prior to therapy. Physical examination and measurements of tumor size were recorded every 2 weeks.

A two-syringe scalp vein technique was initially employed for drug administration. Instances of extravasation resulted in local induration, erythema, and skin necrosis so the technique was changed to injection of the drug by iv push through the tubing of a solution of 5% dextrose in water. Treatment was temporarily withheld if abnormal renal function developed. After this toxicity cleared, treatment was either reinstituted at the same dose level or the study was terminated.

Of the 43 patients entered in this study, 21 had adenocarcinoma (lung [eight], colon [six], stomach [four], and one each of the breast, kidney, and endometrium); 12 had squamous cell carcinoma (lung [seven], cervix [three], and one each of the pharynx and mandible); and five had lymphoma (non-Hodgkin's disease [three, two with poorly differentiated lymphocytic diffuse and one with well-differentiated lymphocytic diffuse] and Hodgkin's disease [two, both with mixed cell type]).

RESULTS: TOXICITY

The toxicity experience is summarized in table 1. Standard Western Cancer Study Group (WCSG) toxicity criteria were used for classification of severity (10). Nausea and vomiting (19 of 43 patients) was not dose related except when the patients were grouped. Those who received $\leq$ 0.9 mg/m^2 had a lower incidence (seven of 20 patients) than those who received higher doses (12 of 23 patients). The symptoms were usually mild, and most frequently occurred with the first treatment. Symptoms were less severe with repeated injections.

Necrotic skin lesions (six patients) were a result of extravasation of small amounts of chromomycin A_3 and all of these instances occurred early in the study. After the technique of administration was changed to iv push through the tubing of a 5% dextrose in water solution, no skin reactions developed. Four of the six patients had severe local inflammation and subsequent ulceration at the injection site. Two patients required hospitalization and one required skin grafting. The others healed within 4 weeks by scar formation.

Six patients had transient elevations of SGOT and/or alkaline phosphatase that returned to normal in the rest interval between Days 10 and 29. None of the elevations exceeded 50% of the baseline value. There was one incidence of probable drug fever (dose level 0.8 mg/m^2) which was noted between Days 8 and 15 when the maximum temperature was 101° F. Three patients developed partial alopecia and one had transient prolongation of the partial thromboplastin time (72 seconds) without evidence of bruising or bleeding. One patient was classified as having 1+ central nervous system (CNS) toxicity because of a subjective description of tenseness and a "lost" feeling. This occurred within 1 hour after each injection and lasted for 8 hours. There were no instances of hypocalcemia observed in this study.

Impairment of renal function was documented in 18 of 43 patients. At submaximal doses there was no clear evidence that linked increasing dose level with

TABLE 1.—Toxicity summary

Dose level (mg/m^2)	No. of patients	No. of treatment courses	Toxic effect*						
			Nausea/vomiting			Renal			
			+	++	+++	+	++	+++	Other†
0.7	3	7	0	1	0	0	1	0	1+ fever(1)
0.8	8	18	2	1	0	1	1	0	1+ alopecia(1)
0.9	9	13	1	2	0	2	1	0	1+ pruritis(1)
1.0	5	10	0	1	0	2	0	0	1+ leukopenia(1) 1+ liver(1) 1+ anemia(1) 1+ thrombocytopenia(1)
1.1	3	3	1	2	0	1	0	0	1+liver(2) 1+ alopecia(1)
1.2	3	9	0	1	0	0	0	0	1+ liver(1) 1+ leukopenia(1) 2+ alopecia(1) 1+ CNS(1)
1.3	3	6	2	1	0	0	1	0	2+ anemia(1)
1.4	3	5	1	1	0	1	1	0	1+ anemia(1)
1.5	3	7	1	0	0	2	1	0	1+ coagulation(1)
1.6	3	4	1	0	0	2	0	1	1+ liver(2) 2+ thrombocytopenia(1) 1+ leukopenia(1)
Totals	43	82	9	10	0	11	6	1	

*+ = mild; ++ = moderate; +++ = severe.
†No. of patients in parentheses.

increasing toxicity. Eight of 25 patients treated at dose levels ≤ 1.0 mg/m^2 developed some degree of renal dysfunction. All instances were either transient or associated with tumor growth and complications. There were only two instances of renal toxicity among nine patients (18 treatment courses) at dose levels from 1.1 to 1.3 mg/m^2. One of these (dose level 1.1 mg/m^2) represents a serum creatinine level of 1.8 mg/100 ml on Day 15. The serum creatinine level on Days 1, 8, and 29 did not exceed 1.0 mg/100 ml. All BUN values were normal and a creatinine clearance obtained on the same day (Day 15) was 90 ml/minute. The second patient received two injections of chromomycin A_3 (dose level 1.2 mg/m^2) and on Day 5 was noted to have a BUN level of 25 mg/100 ml, a serum creatinine level of 2.1 mg/100 ml, and a decrease in creatinine clearance to 54 ml/minute. The patient died 4 weeks later of widespread carcinoma of the colon with tumor involvement of the kidneys but without evidence of tubular necrosis.

At dose levels > 1.3 mg/m^2 the appearance of compromised renal function was noted in eight of nine patients. Seven of these patients developed abnormal renal function during the first course of therapy. These abnormalities were detected on Day 3 (one patient), Day 5 (two patients), Day 8 (two patients), Day 10 (one patient), and Day 15 (one patient). The renal abnormalities persisted for periods of 10–210 days (median, 24 days; mean, 64 days). Re-treatment of two patients who had initial recovery (19 and 21 days) reproduced the abnormalities in renal function for 15 and 50 days respectively. The eighth patient did not develop renal toxicity until Day 5 of the fourth treatment course.

There was no correlation between the development of renal toxicity and previous radiation therapy. Eight patients had received previous chemotherapy with either methotrexate or *cis*-dichlorodiammineplatinum(II). Seven of these eight developed renal toxicity. Total doses of methotrexate varied between 129 and 800 mg. Cyclophosphamide had been given to six patients and all developed renal toxicity after receiving chromomycin A_3. All six patients had also received methotrexate. Caution should be exercised in future studies using chromomycin A_3 in patients who have received any of these agents.

There was a general correlation between the BUN, creatinine, and creatinine clearance studies. Because of the inconvenience of performing creatinine clearance tests and the difficulty in performing

such a test on outpatients, it is felt that future studies could be done without the clinical necessity of monitoring creatinine clearance.

Calcium clearance studies (fig 1) were performed in six patients treated at dose levels of ≥ 1.1 mg/m^2. There was a marked drop noted by Day 10, but the clearance returned toward normal thereafter. Four patients had normal calcium clearance by Day 22 and the other two had returned to within 25% of normal by that same time. The depression of calcium clearance, while uniform, did not persist as long as other renal abnormalities. The effect of single injections of chromomycin A_3 was not studied.

Analysis of the laboratory data obtained during therapy showed no trends in the leukocyte and differential wbc counts, absolute leukocyte or lymphocyte values, platelet count, hemoglobin, bilirubin, alkaline phosphatase, SGOT, LDH, CPK, uric acid, serum calcium, or prothrombin and partial thromboplastin times. No abnormalities in bilirubin, CPK, uric acid, calcium, phosphorus, sodium, potassium, chloride, prothrombin time, serum protein electrophoresis, or ECG could be attributed to chromomycin A_3.

THERAPEUTIC RESPONSE

Although this study was not primarily designed to measure tumor response, all 43 patients had measurable disease parameters. The standard WCSG Solid Tumor Response Criteria were used (10) where objective responses were classified as complete (I-C), partial (I-B), or minor (I-A) response. At dose levels of ≤ 1.0 mg/m^2 (25 patients), there was only one I-B response (Hodgkin's disease, mixed cell type) lasting for slightly > 1 month. Minimal tumor response was also recorded in one patient with poorly differentiated diffuse lymphocytic lymphoma, one with endometrial carcinoma, one with squamous cell carcinoma of the lung, and one with carcinoma of the stomach. None of these minimal responses qualified for I-A responses because of insufficient duration of response or the magnitude of tumor reduction ($< 25\%$ tumor shrinkage required).

At dose levels of ≥ 1.1 mg/m^2 (18 patients), there were two I-B responses (adenocarcinoma of the lung and malignant melanoma) and one I-A response (embryonal rhabdomyosarcoma). All three responses were of short duration (6, 8, and 5 weeks respectively). In addition, there were minimal responses in seven other patients (three with adenocarcinoma of the colon, two with squamous cell carcinoma of the lung, one with nodular lymphohistiocytic lymphoma, and one with squamous cell carcinoma of the cervix).

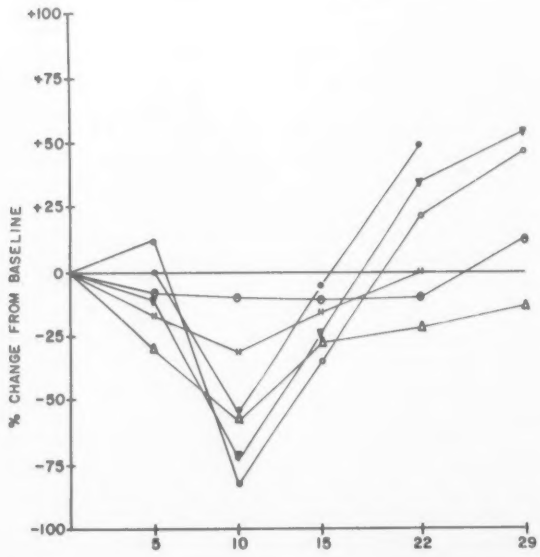

FIGURE 1.—Influence of alternate-day doses of chromomycin A_3 (dose levels of 1.1–1.6 mg/m^2) on calcium clearance. Chromomycin A_3 was given on Days 1, 3, 5, 8, and 10 of a 28-day treatment cycle.

DISCUSSION

This alternate-day treatment program of chromomycin A_3 has shown that larger doses may be given than those tolerated in previous daily dose schedule studies. Neither mucositis nor hematologic toxicity occurred. Renal toxicity continues to be the limiting factor in the use of this agent, but dose levels of 1.3 mg/m^2 for five doses were found to be well tolerated in previously untreated patients. The increased tolerance for individual doses of chromomycin A_3 by this method of administration appears to parallel the clinical experience with the analog mithramycin. The apparent increased risk of chromomycin A_3 renal toxicity in patients previously treated with methotrexate or *cis*-dichlorodiammineplatinum(II) (and perhaps high-dose cyclophosphamide) would indicate the need for special caution in patients previously treated with these potentially nephrotoxic agents. Pre-treatment serum creatinine or BUN tests are required in order to alert the physician to early renal toxicity.

Re-treatment of patients with mild transient renal toxicity was found to reproduce the initial abnormalities but did not appreciably increase the severity or duration of the toxicity.

There were significant responses noted in four patients. I-B responses were found in patients with adenocarcinoma of the lung, malignant melanoma,

and Hodgkin's disease. A single I-A response was found in a patient with embryonal rhabdomyosarcoma. Three of these responses were obtained at doses that exceeded any levels achieved successfully in other phase I trials (7). The WCSG is currently investigating phase II trials at the 1.3-mg/m^2 dose level. If the renal toxicity is found to be tolerable, additional studies may be warranted.

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Acute Nonlymphocytic Leukemia Associated With Nitrosourea Chemotherapy: Report of Two Cases[1]

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SUMMARY

Acute nonlymphocytic leukemia developed in two patients who were treated postoperatively with cytotoxic chemotherapy. Both patients survived > 4 years after the diagnosis of brain tumor, and both had extended periods of myelosuppression secondary to nitrosourea chemotherapy. Nitrosourea therapy may have facilitated the development of acute leukemia in these patients.

[Cancer Treat Rep 60:1257–1261, 1976]

The suspicion that certain chemical agents and therapeutic drugs are capable of producing human leukemia has been widely discussed. Many cases of suspected benzene-induced acute leukemia have been reported (1–4), and chloramphenicol (5–9) and phenylbutazone (10–12) have also been implicated as leukemogenic agents (13). Melphalan therapy for multiple myeloma has also been suspected as being leukemogenic (14–16), while other authors believe that leukemia in this circumstance may be part of the natural history of multiple myeloma in patients who are surviving longer (17). Whether or not these drugs are capable of inducing leukemia, or whether the diseases are themselves pre-leukemic states which follow a natural progression to leukemia with lengthened survival, the association of leukemia in patients treated with cytotoxic agents exists. We have had the opportunity to follow two patients who received nitrosourea chemotherapy for central nervous system neoplasms and who subsequently developed acute nonlymphocytic leukemia.

CASE REPORTS

Patient No. 1

This 56-year-old caucasian woman had surgical removal of an angioblastic meningioma in February 1968, and received postoperative cranial irradiation. She progressed well until January 1970, when she had a seizure and was started on chemotherapy with BCNU, receiving 120 mg/m² on January 7 and 8, February 23 and 24, and April 8 and 9, 1970, without significant myelosuppression. Because of an apparent worsening of her brain scan, BCNU was discontinued and she was treated with oral CCNU at 150 mg/m² on September 23, 1970. Improvement was observed in her speech and gait and no myelotoxicity occurred. Lumbar puncture and bone marrow aspirates were both normal. Four subsequent courses of oral CCNU were administered over the next 7 months at reduced doses and finally, because of severe myelosuppression, CCNU was discontinued.

On July 2, 1971, the bone marrow aspirate showed mild hypocellularity but normal cellular maturation, and the patient was started on VM-26 at 150 mg/m²/week. Over the next 2 years there was no significant change in the patient's condition or in her brain scans and chemotherapy was discontinued on October 18, 1973. In all, the patient received 720 mg/m² of BCNU, 700 mg/m² of CCNU, and 10,000 mg/m² of VM-26.

On December 3, 1974, the patient fell at home and became comatose the next day. She was admitted to the hospital and was found to be pancytopenic. Cerebral arteriogram revealed a small subdural hematoma and vascular mass in the left frontal area, the same location as her previous brain tumor. The cause of her coma, however, was severe hyponatremia, which responded well to conservative management, and her mental status improved. Bone marrow aspirate revealed a hypercellular marrow with

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an abundance of myeloblasts, and the diagnosis of acute myelomonocytic leukemia was made.

The patient received a combination of daunorubicin (45 mg/m²/day given rapidly iv for 3 days) and cytosine arabinoside (100 mg/m²/day in a continuous 72-hour infusion). On January 25, 1975, following one course of chemotherapy, the patient achieved a CR which lasted 1 month. The patient's blast count increased dramatically and on February 25, 1975, marrow aspirate confirmed relapse. The following day the patient developed phlegmasia cerulea dolens of the left leg and died.

Autopsy of Patient No. 1

At autopsy there were small (5 cc) bilateral subdural hematomas. A 3 × 3.5-cm neoplasm was found attached to the left anterior falx. Microscopically, this was identified as an angioblastic meningioma. Although the meninges appeared normal grossly, they were found on microscopic examination to be diffusely invaded by leukemic cells. The general autopsy revealed leukemic filtrates in the marrow, liver, spleen, kidneys, adrenals, and lungs.

Patient No. 2

This 60-year-old caucasian woman had subtotal removal of a left frontal oligodendroglioma on November 21, 1971, and was started on oral methyl-CCNU (meCCNU) at 220 mg/m² 2 months later after a normal lumbar puncture and bone marrow aspirate. She experienced only minimal myelosuppression and received subsequent doses of meCCNU at approximately 6-week intervals until December 1972, when a marrow aspirate showed hypoplasia and megaloblastic changes. Further doses of meCCNU were reduced because of myelotoxicity and finally, after a total meCCNU dose of 2700 mg/m², chemotherapy was discontinued in August 1974.

During the entire year after the cessation of chemotherapy the patient remained pancytopenic (hct level 29%, wbc count 1200 cells/mm³, and platelet count 110,000 cells/mm³). By June 5, 1975, marrow aspirate was hypocellular and there were no megakaryocytes, there were rare immature abnormal cells, and there was a predominance of megaloblastic red cell precursors. The pancytopenia gradually worsened and serial bone marrow aspirates demonstrated a slowly increasing number of myeloblasts. Iron stain of the marrow revealed ring sideroblasts. PAS stain was not taken up by the blasts. Serum B_{12} and folate levels were normal. Sucrose fragility test for paroxysmal nocturnal hemoglobinuria (PNH) was negative. Subsequent marrow aspirates confirmed the diagnosis of acute myelomonocytic leukemia.

The patient had induction therapy for her leukemia, receiving a combination of daunorubicin at 45 mg/m²/day given rapidly iv for 2 days and cytosine arabinoside at 100 mg/m²/day in a continuous 120-hour infusion. However, the patient developed pneumonia with *Candida albicans*, became septic, and died on October 31, 1975.

Autopsy of Patient No. 2

Small (10 cc) recent subdural hemorrhages were present bilaterally at autopsy. Macroscopically, there was no evidence of tumor in the brain or meninges. Microscopic examination of the brain revealed only small focal deposits of oligodendroglial neoplasia in the left and right frontal lobes. There were numerous areas of the brain in which hyphae (*Candida*) were found.

The general autopsy disclosed *Candida* in multiple parenchymal sites as well as in the blood. The marrow was hypoplastic.

DISCUSSION

In the preceding two cases we were able to follow closely the development of acute nonlymphocytic leukemia in patients whose bone marrows had been carefully examined during chemotherapy for brain tumors. There was adequate documentation that marrow aspirates were normal prior to chemotherapy. The first patient received cranial irradiation, but the second did not. The first patient received a moderate dose of the nitrosoureas and a mammoth total dose of VM-26. The second patient received only a large total dose of a nitrosourea. Both patients had long periods of myelosuppression (fig 1).

The drugs that have been associated with the development of acute leukemia are all myelosuppressive. Cohen and Creger (7) suggest that leukemogens may or may not possess similar chemical properties, but all have the capacity to produce an aplastic state. They theorize that leukemia is a rare complication of chloramphenicol-induced aplasia because of the rarity of long survival in aplastic anemia. They report a 39-year-old woman who developed marrow aplasia which responded well to androgenic steroids but finally underwent transformation to nonlymphocytic leukemia 6½ years after the diagnosis of aplastic anemia was made and approximately 2 months before her death. This unusual longevity with aplastic anemia is emphasized by Wolff (18) who found a mortality rate of 50% among 334 patients with aplastic anemia in the first year of their disease. Yunis et al (19) reviewed 46 patients with aplastic anemia and found that of the 38 who died, all were dead by 18 months. Thus, it is likely that those patients who develop aplasia die

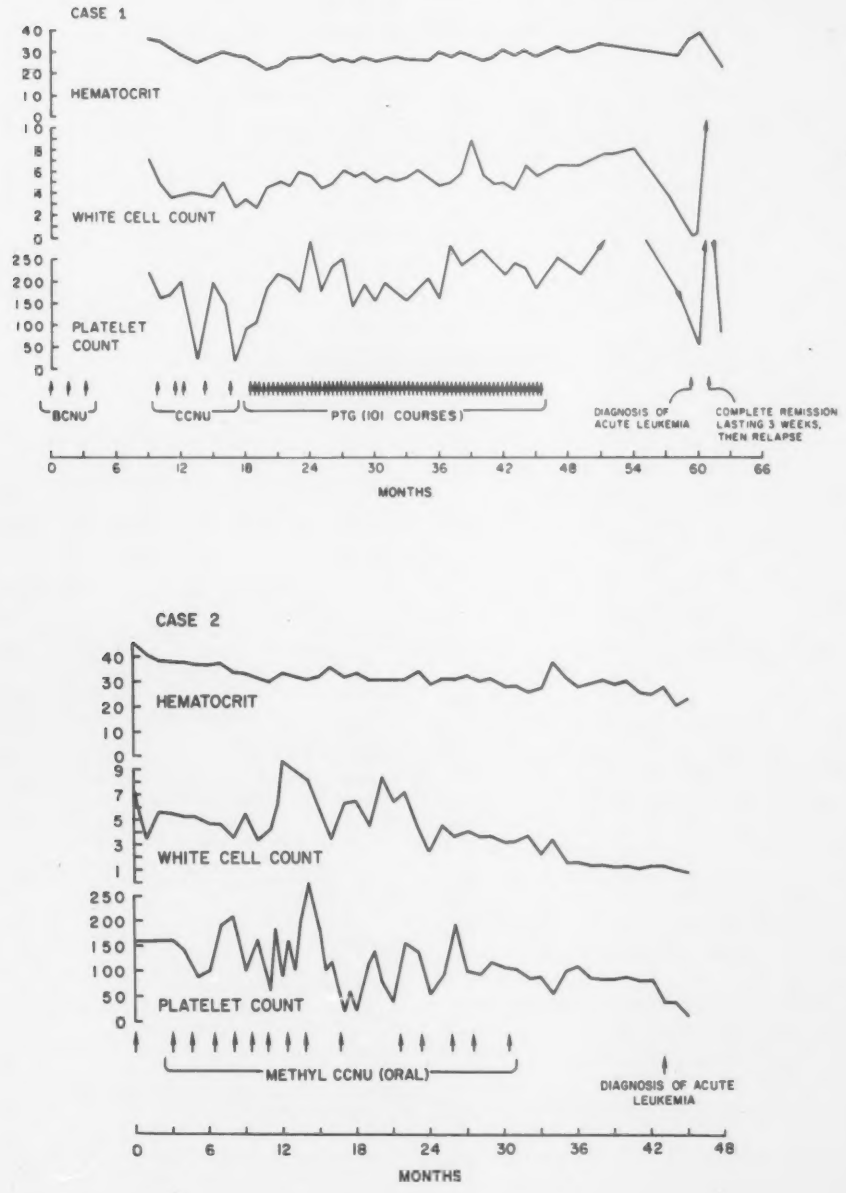

FIGURE 1.—Peripheral blood counts during chemotherapy in 2 patients with brain tumors who developed acute nonlymphocytic leukemia.

from complications of panhypocellularity, while those who survive for several years or more have a greater risk of developing acute leukemia.

Most, if not all, cases of documented chemical leukemogenesis seem to have in common an initial myelosuppressive phase. Marrow suppression appears to be a *sine qua non* of chemical-induced leukemia. Cronkite has said, "Any agent capable of producing aplasia of the bone marrow should be considered potentially leukemogenic" (20). The duration of the period of marrow hypoplasia can be quite variable, sometimes lasting several years. Cases of chloramphenicol-related marrow aplasia have occurred from 2 to 15 years after chloramphenicol treatment, and acute leukemia has evolved after years of marrow aplasia (11). In our two patients the only significant myelosuppression was seen with the nitrosoureas, suggesting strongly that if these cases are examples of drug-induced leukemia, then the responsible drugs in both cases were the nitrosoureas. Even though the nitrosoureas have not been previously associated with the development of human leukemia, the induction of tumors in animals following treatment with a variety of nitrosoureas is well known. MeCCNU is an effective carcinogen in the production of murine neoplasms (21–23); butylnitrosourea and ethylnitrosourea have also been used to induce tumors in rats (24).

Because of the gradual hematologic deterioration in patient No. 2, over a period of several months we were able to closely observe the gradual progression from a hypoplastic marrow state to one with a predominance of monomyeloblasts. This closely parallels other cases of "hypoplastic leukemia," a marrow state which is somehow related to aplastic anemia and to PNH in that both latter marrow states have been observed to interconvert (25) and both have been occasionally observed to end in acute leukemia (26–29). We were unable to document PNH in patient No. 2 since her red cell fragility test was normal on two occasions. We do feel, however, that the clinical course and marrow findings were most consistent with previously described cases of chemically induced leukemia, and that this patient represents one point on a continuum of marrow states which can result from the interruption of normal maturation.

Whether marrow aplasia, "somatic mutation" (13), or viral infection during a chronically immune-suppressed state (30) was finally responsible for these two patients' leukemia, their abnormal marrow states must be considered a complication of chemotherapy. More frequent bone marrow surveillance during chemotherapy might disclose pre-leukemic marrow states which have been thus far overlooked in other patients who have had extended periods of myelosuppression. The need to limit the total dose of chemotherapy in patients who are otherwise well, especially in those being treated on adjuvant therapy protocols, must be examined.

LIST OF COMPOUNDS

BCNU: NSC-409962; CAS reg. No. 154–93–8; urea, 1,3-bis(2-chloroethyl)-1-nitroso-

CCNU: NSC-79037; CAS reg. No. 13010–47–4; urea, 1-(2-chloroethyl)-3-cyclohexyl-1-nitroso-

Cytosine arabinoside: NSC-63878; CAS reg. No. 69–74–9; cytosine, 1-β-D-arabinofuranosyl-, monohydrochloride

Daunorubicin: NSC-82151; CAS reg. No. 23541–50–6

Methyl-CCNU: NSC-95441; CAS reg. No. 33073–59–5; urea, 1-(2-chloroethyl)-3-(4-methylcyclohexyl)-1-nitroso-

VM-26: NSC-122819; 4′-demethylepipodophyllotoxin, 9-(4,6-*O*-2-thenylidene-β-D-glucopyranoside)

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Phase II Study of Vinblastine, Adriamycin, and Procarbazine in Small Cell Carcinoma of the Lung[1,2]

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SUMMARY

Twenty-four patients with small cell carcinoma of the lung were treated with a combination of vinblastine, 5 mg/m² iv on Day 1; adriamycin, 40 mg/m² iv on Day 1; and procarbazine, 100 mg/m² orally on Days 1–7. The courses were repeated every 21 days. Tumor regression was noted in five of eight previously untreated patients, in two of six patients with previous chemotherapy, and in one of ten patients with previous chemotherapy and irradiation. The median duration of response was 130 days (range, 42–488+ days). The major toxic effects were bone marrow depression, gastrointestinal disturbances, and alopecia. This drug combination deserves consideration for inclusion into sequential combination chemotherapy regimens used in the treatment of this tumor type.

[Cancer Treat Rep 60:1263–1266, 1976]

Recent studies indicate an important role for combination chemotherapy in the treatment of small cell carcinoma of the lung (1–7). With various regimens, remission induction is possible in 60%–80% of previously untreated patients. Our current protocols mainly utilize combinations of cyclophosphamide, methotrexate, CCNU, and vincristine, with or without radiotherapy (5). The relapse rate is, however, distressingly high, and there is a clear need for more efficient therapeutic programs. Possible ways for further progress include the development of new drug combinations without cross-resistance to present regimens. This study was designed to evaluate a combination of vinblastine, adriamycin, and procarbazine, three other agents with activity in small cell carcinoma.

MATERIALS AND METHODS

Between January and August of 1975, 24 patients were included in the study. Admission criteria consisted of a histologic diagnosis of small cell carcinoma of the lung and no prior treatment with vinblastine, adriamycin, or procarbazine. Measurable tumors or other lesions enabling decisions about a possible tumor response were present. To avoid additive toxicity, an interval of at least 6 weeks after treatment with CCNU and 3 weeks after the administration of other chemotherapeutic agents was required.

For the purpose of staging, a biopsy from the posterior iliac crest was performed in all patients. In newly diagnosed cases, a bone scan with ^{99m}Tc-diphosphonate and an Anger camera was routinely made in one center (patients 3, 4, 7, and 9–11) and peritoneoscopy with liver biopsy in the other (patients 18 and 19). Other investigations were made when clinically indicated. If the detectable tumor was limited to one hemithorax including supraclavicular lymph nodes, the disease was classified as regional; otherwise the disease was classified as extensive.

Before treatment, the following minimum examinations were made: chest roentgenogram, electrocardiogram, hemoglobin level, leukocyte and platelet counts, serum bilirubin, alkaline phosphatase, glutamic oxaloacetic transaminase, lactic dehydrogenase, creatinine, and uric acid. Throughout the treatment period, peripheral blood cell counts were recorded once weekly.

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Patients were treated with vinblastine, 5 mg/m² iv on Day 1; adriamycin, 40 mg/m² iv on Day 1; and procarbazine, 100 mg/m² orally on Days 1–7. After 21 days, a new treatment cycle was begun. Therapy was terminated if signs of progression occurred. If a wbc count was between 3000 and 5000 cells/mm³ or a platelet count was between 75,000 and 100,000 platelets/mm³, the dosage of all drugs was reduced to three fourths of the amount given in the preceding cycle. With a wbc count < 3000 cells/mm³ and/or a platelet count < 75,000 platelets/mm³, the same dosage reduction was employed but treatment was postponed until the counts exceeded 3000 cells/mm³ and 75,000 platelets/mm³, respectively. The dosage of all drugs was increased by one fourth of the preceding dose if, after two cycles with the same dose level, leukocyte and platelet counts were > 5000 cells/mm³ and 100,000 platelets/mm³, respectively.

The cumulative dose of adriamycin did not exceed 550 mg/m². If this level was reached, adriamycin was discontinued and treatment was continued with only vinblastine and procarbazine. After omission of adriamycin, dose levels of vinblastine and procarbazine were left unchanged and adjustments were made in the subsequent treatment cycles according to the blood cell counts as outlined above.

Beside hematologic effects of treatment, the occurrence of vomiting, infection, bleeding, oral mucositis, liver damage, alopecia, neurologic disturbances, or other signs of toxicity was recorded and rated according to a six-point scale: 0 = none, 1 = slight, 2 = moderate, 3 = pronounced, 4 = life-threatening, and 5 = lethal.

Partial response (PR) was defined as a decrease of > 50% in the transverse area of all measurable lesions, lasting for at least 1 month, without progression elsewhere and without the appearance of new metastases. Complete response (CR) was equivalent to the disappearance of all evidence of disease. A patient was classified as having progressive disease (PD) if any measurable lesion had increased by > 25% or if any new lesion developed. The term no change (NC) was applied to patients who did not fulfill the criteria for response or progression after two cycles of treatment and to patients whose treatment was terminated earlier without signs of response or progression.

RESULTS

Treatment results and toxicity are summarized in table 1. Thirteen patients had regional disease and 11 had extensive disease. CR or PR was obtained in five of eight previously untreated patients, in two of six patients with prior chemotherapy, and in one of ten patients with previous irradiation and chemotherapy. Five of 13 patients with regional disease responded, compared to three of 11 patients with extensive disease. In all but two responders, regression was observed within 21 days from the start of treatment. The median duration of regression was 130 days (range, 42–488+ days). In patients with prior therapy, the responses were comparatively brief (49, 53, and 148 days). The same observation is apparent for patients with extensive disease (42, 49, and 112 days).

Dose reduction was performed in the first cycle of treatment in five patients with prior irradiation and chemotherapy and in one patient with prior chemotherapy. Escalation of initial dosage in later treatment cycles was made in only two of 24 patients. In 20% of the noninitial treatment cycles, the time interval from the preceding cycle was > 3 weeks.

The main hematologic side effect was leukopenia. Two cases of nonfatal infections were encountered, septicemia in one patient and pneumonia in another. Platelet counts < 100,000 platelets/mm³ were recorded in five patients, all having had prior drug treatment with or without radiotherapy. No bleeding was noted and no platelet transfusions were required. Anemia necessitating transfusions of packed red blood cells was seen in six patients, four having had prior irradiation and chemotherapy.

Nausea and vomiting occurred in all but a few patients and was mainly related to procarbazine but did not lead to interruption of therapy. One patient (No. 11) died during remission as a result of widespread herpes zoster. Mild to pronounced alopecia was noted in most patients. There was no oral mucositis or liver toxicity, and no adriamycin cardiomyopathy was seen.

DISCUSSION

In small cell carcinoma of the lung, various combinations using adriamycin and other chemotherapeutic agents have been investigated, with a response rate exceeding 75% (2,3,7). In all of these studies, cyclophosphamide was one of the component drugs. One reason for the choice of this agent has been the proved synergism between cyclophosphamide and adriamycin in certain experimental tumor systems (8).

Our combination of vinblastine, adriamycin, and procarbazine was designed as an alternative to our present program, consisting of cyclophosphamide, methotrexate, CCNU, and vincristine, to be used in relapse cases or for possible inclusion in future combination chemotherapy programs.

The activity of adriamycin and procarbazine in

TABLE 1.—Patients with small cell carcinoma of the lung treated with vinblastine, adriamycin, and procarbazine (VAP)

Patient No.	Age/sex	Disease stage*	Performance status†	Prior therapy‡	Duration of VAP treatment (days)	Response	Day of regression	Duration of regression (days)	Hematologic toxicity§
1	65/M	E	40	CTX, CCNU, VCR	25	NC	—	—	+ +
2	43/M	R	80	CTX, CCNU, MTX, VCR	95	PR	42	53	+++
3	58/M	E	70	None	63	PR	21	42	++
4	72/M	E	70	None	21	PD	—	—	+
5	62/M	R	60	CTX, CCNU, VCR	34	NC	—	—	++
6	53/F	R	60	CTX, CCNU, MTX, VCR	169	PR	21	148	++++
7	62/M	R	70	None	196	CR	21	175	++++
8	62/M	R	70	CTX, CCNU, VCR	124	NC	—	—	+++
9	72/F	R	90	None	334	NC	—	—	+++
10	69/M	R	40	None	509+	CR	21	488+	+++
11	61/F	R	70	None	177	PR	21	156	+++
12	64/M	R	70	CTX, CCNU, MTX, irradiation	92	NC	—	—	++
13	71/M	E	50	CTX, CCNU, VCR, MTX, irradiation	132	NC	—	—	++
14	66/M	E	70	CTX, CCNU, MTX, irradiation	42	NC	—	—	+
15	55/F	R	60	CTX, CCNU, MTX, irradiation	28	PD	—	—	+++
16	66/M	R	60	CTX, CCNU, MTX, VCR, irradiation	77	NC	—	—	++++
17	59/M	E	60	CTX, CCNU, MTX, irradiation	54	PD	—	—	++
18	74/M	E	80	None	42	NC	—	—	++++
19	73/M	E	70	None	124	PR	12	112	+
20	67/M	R	60	CTX, CCNU, MTX, irradiation	26	NR	9	17	+++
21	61/F	R	70	CTX, CCNU, MTX, irradiation	91	PD	—	—	++
22	50/F	E	50	CTX, CCNU, MTX, VCR, irradiation	90	PR	41	49	+
23	70/M	E	60	CTX, CCNU, MTX, VCR	77	NC	—	—	+
24	58/M	E	60	CTX, CCNU, MTX, VCR, irradiation	33	PD	—	—	++++

*E = extensive; R = regional.

†According to Karnofsky scale.

‡CTX = cyclophosphamide; MTX = methotrexate; VCR = vincristine.

§+ = lowest wbc count 3000–5000 and/or lowest platelet count 75,000–100,000; ++ = lowest wbc count 2000–3000 and/or lowest platelet count 50,000–75,000; +++ = lowest wbc count 1000–2000 and/or lowest platelet count 25,000–50,000; ++++ = lowest wbc count < 1000 and/or lowest platelet count < 25,000.

small cell carcinoma is well established. Cumulated data yield a response rate of 28% and 25%, respectively, for these drugs as single agents (9,10). Only scanty information is available for vinblastine. The related drug, vincristine, is known to be active in small cell carcinoma (11). In the study by Crosbie et al (12), a slight difference in survival in favor of vinblastine was noted between oat-cell cancer patients treated with this drug and control cases; however, the small number of patients precludes definite conclusions.

In this study, responses in five of eight previously untreated patients and seven of 14 patients not having prior radiotherapy are encouraging and indi-

cate that the combination may be more effective than any one of the single component agents. Direct comparison with other studies of adriamycin combinations is not possible.

In patients with prior radiotherapy, the effect of the combination was unimpressive. Leukopenia at the start of treatment, which necessitated initial dose reduction, may, in part, explain the poor results in this group. The patients with prior chemotherapy but no prior irradiation therapy are few and no conclusions concerning cross-resistance between the different drugs are possible. In malignant lymphoma, vincristine and vinblastine are supposed to be non-cross-resistant (13). Cross-resistance between vinca alkaloids and anthracycline antibiotics has been described in some experimental tumor systems (8). The clinical significance of these findings in small cell carcinoma is, however, not known.

In conclusion, we feel that the combination of vinblastine, adriamycin, and procarbazine merits further evaluation and could be considered for integration into sequential multi-drug regimens for the treatment of small cell anaplastic carcinoma of the lung.

LIST OF COMPOUNDS

Adriamycin: NSC-123127; CAS reg. No. 23214-92-8

CCNU: NSC-79037; CAS reg. No. 13010-47-4; urea, 1-(2-chloroethyl)-3-cyclohexyl-1-nitroso-

Cyclophosphamide: NSC-26271; CAS reg. No. 6055-19-2; 2*H*-1,3,2-oxazaphosphorine, 2-[bis(2-chloroethyl)amino]tetrahydro-, 2-oxide, monohydrate

Methotrexate: NSC-740; CAS reg. No. 59-05-2; glutamic acid, *N*-[*p*-[[(2,4-diamino-6-pteridinyl)methyl]methylamino]benzoyl]-

Procarbazine: NSC-77213; CAS reg. No. 491-88-3; *p*-toluamide, *N*-isopropyl-α-(2-methylhydrazino)-, monohydrochloride

Vinblastine: NSC-49842; CAS reg. No. 6449-03-2; vincaleukoblastine, sulfate (1:1), monohydrate

Vincristine: NSC-67574; CAS reg. No. 2068-78-2; leurocristine, sulfate (1:1)

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Randomized Comparison of Melphalan and 5-Fluorouracil in the Treatment of Advanced Gastrointestinal Cancer[1,2,3]

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Melphalan is a phenylalanine derivative of mechlorethamine. It was originally synthesized in the hope that, as an analog of phenylalanine mustard, it might be selectively metabolized in melanoma cells. It has been found to be active against a number of experimental tumors including the Walker 256 carcinosarcoma, IRC741 leukemia, T8 Guérin epithelioma, Ehrlich carcinoma, and S91 melanoma.

Clinical evaluation has been largely restricted to patients with multiple myeloma, malignant melanoma, and ovarian carcinoma. In this regard, melphalan has been shown to be an effective agent in the treatment of some solid tumors, but it has not yet been fully evaluated in randomized comparative trials of patients with gastrointestinal (GI) cancer. In this study we have compared the effect of melphalan and 5-fluorouracil (5-FU), both given as intermittent therapy for the treatment of advanced GI cancer in a prospective randomized trial.

MATERIALS AND METHODS

Patient Characteristics

Sixty-five patients with histologically proven carcinoma originating in the GI tract without any hope of surgical cure were entered into the trial. Twenty-two patients had gastric cancer (mean age, 60.2 years; range, 36–73 years), 33 had colorectal cancer (mean age, 60.3 years; range, 38–76 years), and ten had pancreatic or biliary tract cancer. All of the patients had measurable areas of known malignant disease and these areas served as objective indicators of the response to chemotherapy.

There were 45 men and 20 women in this study. All of the patients were ambulatory and had an estimated survival of $\geq$ 6 weeks. No patient had received previous cytotoxic therapy or radiotherapy and all had wbc counts $>$ 4.0 cells $\times$ 10^3/mm^3 and platelet counts $>$ 150 platelets $\times$ 10^3/mm^3. Chemotherapy was deferred for 4 weeks after any abdominal operation.

All patients except one were evaluable at 6 weeks to determine the response to therapy. One patient died 2 weeks after starting treatment with 5-FU and this death was assumed to be drug related. The sites of the primary tumors and the indicator lesions are shown in table 1, together with the histologic cell type and grading. All patients were symptomatic from their malignant disease and the performance status (PS) prior to therapy varied from 0 to 2. All patients came to the outpatient clinic for treatment and subsequent investigation.

Treatment Procedure

Patients were randomized to treatment with either melphalan or 5-FU. Melphalan was administered orally in tablet form at a dose of 6 mg/m^2/day for 5 days. 5-FU was administered by rapid iv injection at a dose of 500 mg/m^2/day for 5 days. Treatment was interrupted earlier if specific signs of toxicity were observed. When a second course was given the dosage of either drug was reduced if significant toxicity was observed with the first course.

Hemoglobin levels and leukocyte and platelet counts were estimated on Days 1, 3, and 5 of treatment and at weekly intervals thereafter. Patients were reassessed 6 weeks after starting treatment and then received a further course of cytotoxic therapy. All patients whose tumor remained objectively stable or who showed an objective response received the same drug previously given. When progression of the malignant disease occurred the patient received the alternative treatment arm.

Assessment of Response

Objective response was defined as a reduction by $\geq$ 50% of the product of the two longest perpendicu-

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[2]Supported by contract N01-CM-43727 from the Division of Cancer Treatment, National Cancer Institute, National Institutes of Health, Department of Health, Education, and Welfare.

[3]Melphalan: NSC-8806; CAS reg. No. 3223-07-2; alanine, 3-[*p*-[bis(2-chloroethyl)amino]phenyl]-, monohydrochloride, L-.

5-Fluorouracil: NSC-19893; CAS reg. No. 51-21-8.

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TABLE 1.—Histologic appearance of primary tumor and site of indicator lesions in patients treated with melphalan and 5-FU

Primary tumor	Initial treatment	Mean age (yrs)	Histologic appearance			Site of indicator lesions			
			Well differentiated	Moderately differentiated	Anaplastic	Primary tumor	Liver	Intraperitoneal tumor	Lung
Stomach	Melphalan	60.6	2	4	10	9	4	3	0
	5-FU	59.3	1	4	1	2	1	2	1
Colon	Melphalan	59	3	5	1	2	7	7	0
	5-FU	55.6	1	10	5	5	4	2	0
Rectum	Melphalan	64.5	2	1	2	0	1	2	1
	5-FU	57.5	1	1	1	0	1	3	0
Pancreas and biliary tract	Melphalan	56.7	0	3	0	1	1	1	0
	5-FU	56.7	2	3	2	0	7	0	0

lar diameters of the most clearly measurable tumor. If the liver was the primary indicator lesion, a reduction by ≥ 30% of the sum of the measurements below each costal margin in the mid-clavicular lines and the xiphoid process with improvement of all pretreatment abnormalities of liver function constituted an objective response. Patients in whom the indicator lesion regressed but new lesions appeared were said to have disease progression. Objective stasis was judged to have occurred when there was insufficient regression to meet the above criteria or when there was < 25% increase in any measurable lesion.

A patient was judged to have progressive disease when the increase in any measurable lesion was > 25%, when any new areas of malignant disease appeared, or if there was significant deterioration in symptomatology or PS.

In addition to this objective assessment an attempt was made to assess palliation in terms of relief of pain or improvement in PS. The patients were graded according to the following criteria: 0 = normal activity, 1 = ambulatory but with some restrictions, 2 = in bed < 50% of the time, 3 = in bed > 50% of the time, and 4 = bedridden or terminal.

RESULTS

Toxic Effects

In evaluating both treatment arms, a total of 57 courses of 5-FU and 91 courses of melphalan were given and the toxic effects of these are listed in table 2. Nausea and vomiting usually began the day after the first dose of cytotoxic agent and continued from 1 to 3 days. Nausea and vomiting were not severe with either drug but were least severe with melphalan. Diarrhea was uncommon with both drugs but oral conditions (aphthous ulceration, glossitis, or oral thrush) were more common with 5-FU.

Marrow depression occurred in approximately one third of the courses of 5-FU and in one fourth of the courses of melphalan with evidence of cumulative toxicity in approximately 5%. One patient died 2 weeks after receiving 5-FU with profound leukopenia and thrombocytopenia; the death is presumed to be drug related. There was no evidence of nephrotoxicity or hepatotoxicity.

Therapeutic Results

The objective responses to therapy are shown in table 3. When given as a first treatment arm (32 patients) melphalan produced an objective remission in four (12.5%), stasis in eight (25%), and progressive disease in the remaining 20 (62.5%). Thirteen of these patients (40.6%) received second courses of melphalan resulting in a further remission in one patient, progression in seven, and stasis in five. Only six patients received more than two courses of melphalan when given as the initial treatment. When given as a second treatment arm (14 patients) melphalan produced a remission in two (14.3%), stasis in five (35.7%), and progressive disease in seven (50%). Seven patients then received further courses of melphalan achieving stasis in five, but two progressed and there were no further objective responses. Five patients received more than two courses of melphalan when given as a second treatment arm; two of these patients have now received seven and eight courses respectively over a period of 1 year and both are well. The other patients with objective remissions were less controlled and the median duration of the response was 3.0 months (1.5–6 months). Of the seven patients with an objective response, the liver was the indicator lesion in five.

5-FU given as a first treatment (33 patients) produced an objective response in five patients (15.2%), stasis in nine (27.3%), and progressive disease in the remaining 18 (54.5%) (table 3). One patient died at 2 weeks and was not objectively assessed.

Ten of these patients received a second course of 5-FU resulting in an objective remission in one (10%), stasis in four (40%), and progression in five (50%). Four patients received more than two courses of 5-FU when given as a first treatment arm. When given as a second treatment arm (seven patients) after progression on melphalan therapy, 5-FU failed to produce stasis or remission and all patients progressed. One patient received a second course based on excellent palliative results and had further progression.

TABLE 2.—Toxic effects

Toxic effect	5-FU (% of courses)	Melphalan (% of courses)
Nausea and/or vomiting	39	17
Diarrhea	5	2
Sore mouth	14	2
Skin rash	2	1
Leukopenia (< 1.0 cells × 10^3/mm³)	14	9
Thrombocytopenia (< 100 platelets × 10^3/mm³)	11	13
Anemia (fall in hemoglobin level > 2 g/100 ml)	9	2
Cumulative toxicity (% of patients)	5	4

Palliation

There are difficulties in the assessment of palliation in trials in which there is movement from one treatment arm to another. An evaluation done at the end of the first treatment arm suggested worthwhile palliation in 13 of 31 patients (41%) receiving melphalan and in 14 of 33 patients (42%) receiving 5-FU. Additionally, eight of 14 patients (57%) receiving melphalan and two of seven receiving 5-FU as a second treatment arm were also improved.

Overall, the mean duration of symptomatic palliation was 3.5 months (range, 1.5–6.0 months) for gastric cancer, 6.5 months (range, 3.0–16 months) for colorectal cancer, and 6.5 months (range, 2–9 months) for pancreatic and biliary cancer.

DISCUSSION

For several years now the use of melphalan in solid tumor therapy has been largely restricted to melanomas, sarcomas, and ovarian carcinomas. However, recent reports from Taylor et al (1) suggest that melphalan may have an effect in advanced breast cancer with 22 responses in 91 patients including three CRs. Fisher et al (2) have reported the early results of a multicenter trial using melphalan as adjuvant therapy for patients with stage II breast cancer. There would appear to be evidence that patients treated with melphalan have a reduced early failure rate particularly if premenopausal.

There is little documentation of the value of melphalan in the treatment of advanced GI cancer. Rochlin et al (3) advocated the use of melphalan as a drug of "second choice" in GI cancer even though they achieved only 7% and 1% response rates for colonic and gastric cancer respectively. Carter and Friedman (4) in their review article reported a 15% response rate for large bowel cancer with a daily oral dose of melphalan and a 25% response rate when used as weekly maintenance therapy after an initial loading dose.

The results of the present trial would appear to confirm that melphalan given orally and intermittently in 5-day courses every 6 weeks may be active against advanced GI cancer. Of 22 patients with colorectal cancer receiving melphalan, five (23%) had objective remissions compared with one objective remission in 18 patients with gastric cancer identically treated. A patient with gallbladder cancer also had an objective remission.

5-FU has been the most extensively studied drug in GI cancer with a response rate of 21% in > 2000 patients (4) and is, therefore, the standard against which other drugs may be compared. Our results with 5-FU (15%) are comparable to some other series although the response rate is slightly lower than the overall mean reported by Carter and Friedman (4). It does suggest that the criteria of patient entry into this trial were similar to those of the previously reported studies.

Used as the initial cytotoxic agent we have found that melphalan is similar in efficacy to 5-FU but has a more convenient method of administration and requires, therefore, fewer outpatient visits by the patient. Additionally, melphalan has proved to have fewer unwanted toxic effects and those that did occur were less severe than those with 5-FU. The palliative effects of both drugs were very similar.

CONCLUSION

This study confirms that melphalan appears to have activity against GI cancer and is comparable to 5-FU. This activity is achieved with a minimum of

TABLE 3.—Initial response to first treatment arm

Drug	Site of primary tumor	No. of patients treated	No. of patients with—		
			Objective response	Disease progression	Stasis
Melphalan	Stomach	16	1	12	3
	Colon	9	1	6	2
	Rectum	4	1	1	2
	Other	3	1	1	1
	Totals	32	4	20	8
5-FU	Stomach	6	1	3	1
	Colon	16	4	9	3
	Rectum	4	0	2	2
	Other	7	0	4	3
	Totals	33*	5	18	9

*1 patient not evaluated.

unwanted toxic effects. The ease of administration minimizes the number of required outpatient attendances. A study of melphalan in combination with other cytotoxic agents would seem to be indicated.

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Combination Chemotherapy With Three Marginally Effective Agents, CCNU, Vincristine, and Bleomycin, in the Treatment of Stage III Melanoma[1,2,3]

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SUMMARY

Forty-two patients with stage III melanoma were treated with a combination of CCNU, bleomycin, and vincristine. Seventeen of 35 evaluable patients achieved an objective response (48.5%) including five patients with CRs. The median duration of response was 4 months (range, 1–18 months). The responders had a median survival of 278 days (range, 78–1100 days) and the nonresponders had a median survival of 168 days (range, 34–619 days). The toxicity of the combination was within an acceptable range with no toxic deaths.

[Cancer Treat Rep 60:1273–1276, 1976]

This study reports a therapeutic trial of disseminated melanoma (stage III) (1) with a combination of CCNU, vincristine, and bleomycin. The study was started in 1972 and the choice of the combination was influenced by the excellent results reported by Moon (2) in a small series of patients treated with BCNU and vincristine. CCNU was chosen instead of BCNU because of its availability at the time of the design of the protocol and the obvious advantage of oral administration. Bleomycin was added for its cutaneous tropism and its lack of additive toxicity in combination with other agents.

MATERIALS AND METHODS

Patient Selection

Forty-two consecutive patients with biopsy-proven recurrent and metastatic unresectable melanoma (stage III) were treated with the triple-drug combination. Patients with brain metastases were excluded. All patients had progressive disease. Some patients were in relapse after previous chemotherapy (11 patients) or immunotherapy (13 patients).

Seven patients were not evaluable for the following reasons: a lack of clear pathologic proof (one patient with hepatic metastases seen by laparoscopy without biopsy; patient still alive after 19 months), a lack of followup (three patients lost to the study on Days 14, 27, and 28), one early death not related to melanoma (Day 26) and one related to melanoma (cardiac arrest on Day 10 due to myocardial metastases), and one treatment refusal (Day 28). A total of 16 men (age range, 17–66 years; median age, 43.5 years) and 26 women (age range, 20–78 years; median age, 60.5 years) were entered in this study.

Of the total of 42 patients, 11 had received prior chemotherapy (ten of the 35 evaluable patients)

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[3]CCNU: NSC-79037; CAS reg. No. 13010-47-4; urea, 1-(2-chloroethyl)-3-cyclohexyl-1-nitroso-.

Vincristine: NSC-67574; CAS reg. No. 2068-78-2; leurocristine, sulfate (1:1).

Bleomycin: NSC-125066; CAS reg. No. 11056-06-7; [2,4′-bithiazole]-4-carboxylic acid, 2′-(2-aminoethyl)-, monohydrate.

[4]Service de Médecine et Laboratoire d'Investigation Clinique, Institut Jules Bordet, Brussels, Belgium (Drs. De Wasch, Bernheim, and Kenis); Département de Chimiothérapie du Centre René Goffin, Centre Hospitalier de Tivoli, IMMS, La Louvière, Belgium (Dr. Michel); and Service de Chirurgie, Institut Jules Bordet and Centre des Tumeurs de l'Université Libre de Bruxelles, Belgium (Dr. Lejeune). These institutions are members of the European Organization for Research on Treatment of Cancer.

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including four who had received CCNU. Thirteen patients had received some form of immunotherapy (BCG, dinitrochlorobenzene, melanoma cells, or levamisole). The disease duration prior to chemotherapy ranged from 2 to 96 months with a median duration of 26 months.

Treatment Regimen

CCNU was given at a dose of 130 mg/m^2 orally every 6 weeks. It was postponed weekly if the leukocyte count was < 4000 cells/mm^3 or the platelet count was < 100,000 platelets/mm^3. Vincristine at a dose of 1.4 mg/m^2 (maximum unit dose, 2 mg) and bleomycin at a dose of 10 mg/m^2 (maximum unit dose, 15 mg) were administered weekly for 20 weeks by the iv route. Vincristine was discontinued for neurologic toxicity if symptoms and signs were more severe than paresthesias and loss of deep tendon reflexes. It was reduced by 50% when paresthesias were disturbing or when patients became constipated. Bleomycin was given for a maximum total dose of ≤ 300 mg and was discontinued with the first signs of pulmonary toxicity. Treatment was given until reactivation or progression of the disease.

Response Criteria

Only objective criteria were used. CR was defined as a disappearance of all manifestations of disease. PR was defined as a regression of ≥ 50% of the initial size of measurable lesions (defined as a reduction by 50% of the sum of the products of the two longest perpendicular diameters of all tumors for at least 1 month) without progression or appearance of any new lesion at any site.

RESULTS

Of 35 evaluable patients, 16 had only cutaneous, subcutaneous, or lymph node (one patient) metastases, two had only one metastatic site confined respectively to the lung and the liver, and 17 had more than one metastatic site (table 1).

All treatment results are summarized in tables 2 and 3. Responses were obtained (PR plus CR) in 17 of 35 evaluable patients (48.5%) with a response rate for all patients of 40.7%. In comparing patients with prior chemotherapy and patients without prior chemotherapy the response rate was 40% (four of ten) and 52% (13 of 25) respectively. The group of patients with prior chemotherapy included three patients treated with and resistant to CCNU alone; in the study protocol one patient achieved a CR, one had a PR, and the third had disease progression. A fourth patient was previously treated with a combination of CCNU and vincristine; no response was observed. The remaining previously treated patients had received either DTIC or melphalan. Taking into account sex distribution, the response rate seems higher in women (13 of 23) than in men (four of 12). Analyzing the response in relation to the different sites of metastases, the five patients with CR were found in the group with only cutaneous, subcutaneous, and/or lymph node dissemination. No response was observed in patients with bone metastases. The PR rate in patients with visceral metastases (41%, seven of 17 patients) was very encouraging.

TABLE 1.—Treatment of stage III melanoma with a combination of CCNU, bleomycin, and vincristine

Metastatic site	Total No. of patients	No. with only 1 metastatic site
Cutaneous, subcutaneous, and lymph nodes	31	16
Visceral (total)	17	2
Lung	12	1
Liver	8	1
Pancreas	1	
Bone	6	
Epidural + cranial nerves	2	

The median survival was 278 days (range, 78–1100 days) for responders and 168 days (range, 34–619 days) for nonresponders. The median response duration was 4 months with a range from 1 to 18 months.

Toxicity data are summarized in tables 4 and 5. Hematologic toxic effects were within an acceptable range; no toxic deaths were observed. Of 34 hematologically evaluable patients five had thrombocytopenia (< 30,000 platelets/mm^3) and four had leukopenia (< 1000 cells/mm^3). In two patients leukopenia was complicated by infection amenable to cure by antibiotics. There was a fall in the hemoglobin level of > 40% in ten patients and 12 had a reduction between 20% and 40%. Gastrointestinal intolerance due to CCNU was observed in almost all patients; alopecia was severe in eight. According to the protocol, bleomycin was stopped twice for lung toxicity. It was reduced for severe skin toxicity in four patients. The dose of vincristine was reduced in 12 patients. A disturbing fact is that a weight loss of > 10% was observed in nine of 20 patients. Even responsive patients experienced this adverse effect of chemotherapy.

TABLE 2.—Treatment of stage III melanoma with a combination of CCNU, bleomycin, and vincristine—response to chemotherapy

	Total No. of patients	No. of evaluable patients	Response		
			Complete	Partial	Progression
No prior chemotherapy	31	25	4	9	12
Prior chemotherapy	11	10	1	3	6
Men	16	12		4	8
Women	26	23	5	8	10
Totals	42	35	5	12	18

TABLE 3.—Treatment of stage III melanoma with a combination of CCNU, bleomycin, and vincristine—response according to site

Metastatic site	Total No. of patients	Response		
		Complete	Partial	Progression
Cutaneous, subcutaneous, and lymph nodes	31	6*	9	16
Visceral (total)	17	—	7	10
Lung	12	—	6	6
Liver	8	—	2	6
Pancreas	1	—	—	1
Bone	6	—	—	6
Cranial nerves	2	—	—	2

*1 patient had CR for cutaneous, subcutaneous, and lymph node metastases but had only PR for lung metastases.

TABLE 4.—Treatment of stage III melanoma with a combination of CCNU, bleomycin, and vincristine—hematologic tolerance*

Leukocyte count (cells/mm³)	Thrombocyte count (platelets/mm³)	Fall in hemoglobin level (%)
> 3000 (11 patients)	> 100,000 (10 patients)	< 20 (12 patients)
1000–3000 (19 patients)	30,000–100,000 (19 patients)	20–40 (12 patients)
< 1000 (4 patients)	< 30,000 (5 patients)	> 40 (10 patients)

*1 patient was not evaluable for hematologic tolerance.

TABLE 5.—Treatment of stage III melanoma with a combination of CCNU, bleomycin, and vincristine—clinical tolerance

Toxic effect	No. of evaluable patients	Degree of toxicity (No. of patients)	
		Moderate	Severe
Gastrointestinal intolerance	34	21	12
Alopecia	34	12	8
Skin	29	7	4*
Neurologic	32	14	12†
Lung	27	2‡	
Weight loss > 10%	20	9	

*Bleomycin dose reduction was required.
†Vincristine dose reduction was required.
‡1 patient had pneumonia and 1 had early pulmonary fibrosis.

DISCUSSION

The response rate obtained with the triple-drug combination is among the highest in published series utilizing the same criteria of response and with a sufficient number of patients (3,4). The good results obtained in our series do not seem influenced by the percentage of patients with only soft tissue deposits since the percentage of objective remissions was not significantly different compared to patients with visceral metastases. The 41% remission rate in patients with visceral involvement other than bone metastases is particularly interesting but might have been favorably influenced by the female predominance. Nevertheless, this therapeutic regimen compares favorably with other reported combinations of chemotherapy (3,4) and even combinations of chemoimmunotherapy (5). For the two-drug combinations with DTIC the highest remission rate was 21%. For the two-drug combinations with the nitrosoureas only Moon (2) had a response rate > 20%. With the triple-drug combinations the highest percentage of response was 63% in a series of 16 patients treated with DTIC, BCNU, and vincristine (6). With the same three drugs the response was only 21% in the recently published trial of the World Health Organization (7), which is not more than the response rate obtained with DTIC alone, the unique chemotherapeutic agent "definitely active" in malignant melanoma (8).

Considering that CCNU, vincristine, and bleomycin used as single agents give remission rates of 13%, 12%, and 0 respectively (8), our results are really striking. One tentative explanation of this success might be that each drug was utilized at its full dosage due to the absence of cumulative toxicity. A similar result was obtained recently with a two-drug combination by the Western Cancer Study Group who concluded that the addition of vincristine significantly affects the objective response rate of melanoma to CCNU (9). Another possibility is that vincristine accumulates cells in mitosis, the most sensitive phase to bleomycin (10). The remissions, including one CR, that we obtained in patients resistant to CCNU alone are another argument in favor of the use of combinations of marginally effective drugs.

The fact that the 4-month median duration of remission corresponds to the nearly 4-month increase in median survival of responders suggests that this treatment may have prolonged life. This should be investigated in a randomized trial.

This study does not permit any conclusion about the possible absence of cross resistance between our combination and DTIC. It is tempting to try to alternate these two regimens, possibly with immunotherapy.

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Hepatic Artery Infusion of 5-FUDR After Prior Systemic 5-Fluorouracil[1,2,3]

Thomas Buroker,* Michael Samson, Juan Correa, Robert Fraile, and V. K. Vaitkevicius[4]

SUMMARY

Twenty-one patients with disseminated colon carcinoma and clinically significant liver metastases were treated with 5-FUDR via hepatic artery infusion (HAI). All patients had previously received systemic chemotherapy consisting of either 5-fluorouracil alone or in combination with other agents. At the time of the initiation of the HAI, clinical disease in all patients was progressing. A PR of hepatic metastases was noted in eight patients (35%) with a median and mean duration of response of 4.5 and 5.0 months respectively. The median and mean survival from the start of HAI for responders was 8.0 and 9.0 months and for nonresponders was 1.0 and 1.6 months respectively. It appears that a significant response rate can be achieved with HAI of 5-FUDR in spite of previous exposure to fluorinated pyrimidines.

[Cancer Treat Rep 60:1277–1279, 1976]

Hepatic artery infusion (HAI) of 5-FUDR has been shown to be an effective method of controlling liver metastases of disseminated gastrointestinal cancer (1–5). A frequent therapeutic dilemma on a university oncology service is the patient who has hepatic metastases from colon cancer and has received systemic 5-fluorouracil (5-FU) prior to his referral. Are the hepatic metastases in these patients refractory to intra-arterial 5-FUDR? Can responsiveness to HAI be predicted based on previous sensitivity to 5-FU? This retrospective analysis was performed to answer these questions since HAI requires a considerable amount of patient and physician time and can be quite cumbersome to patients.

MATERIALS AND METHODS

From 1965 to 1976, 21 patients with colon carcinoma and hepatic metastases were treated with 5-FUDR via HAI after having received systemic 5-FU. All patients had histologically proven malignancy. There were 12 men and nine women with a median age of 54 years (range, 37–73 years). All patients had received an adequate trial of 5-FU systemically. An adequate trial was defined as a minimum of two consecutive courses of 5-FU given to patients with clearly progressive hepatic metastasis in the face of systemic toxicity, ie, severe mucositis and/or myelosuppression. Ten patients received 5-FU at a dose of 12 mg/kg/day iv bolus for 5 consecutive days while 11 patients received 5-FU at a dose of 30 mg/kg as a 24-hour continuous infusion for 5 consecutive days. There was one PR in each 5-FU-treated group. Both forms of treatment were repeated every 4 weeks (6). The 5-FU had been discontinued at least 4 weeks prior to the institution of HAI. In all patients there was pathologic enlargement of the liver with metastatic disease documented by liver biopsy and/or liver scan. The hepatic artery catheter was either inserted transabdominally (7) or transbrachially (8), depending upon the technical feasibility and the patient's overall clinical condition. Nine patients had transabdominal and 12 patients had transbrachial artery catheter placement.

5-FUDR was infused continuously through the catheter via a Watkins pump[5] at a dosage of 0.3 mg/

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[3]5-FUDR: NSC-27640; uridine, 5-fluoro-2'-deoxy-.

5-Fluorouracil: NSC-19893; CAS reg. No. 51–21–8.

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TABLE 1.—Survival data of responders and nonresponders

Patient No.	Duration of disease from treatment to HAI (mos)	Length of remission (mos)	Length of survival from start of HAI (mos)
Responders			
1	8	4	7
2	4	9	15
3	19	2	2
4	9	5	14
5	41	3	7
6	18	2+	3+
7	2	9	9
8*	14	6	15
Mean	14.4	5	9.0
Median	11.5	4.5	8.0
Range	2–41	2–9	2–15
Nonresponders			
1	14		3
2	1		1
3	37		1
4	4		1
5	5		1
6	84		1
7	44		1
8	8		1
9†	15		1
10	12		4
11	24		1
12	12		1
13	4		1
Mean	20.3		1.6
Median	12		1.0
Range	1–84		1–4

*Prior responder to bolus 5-FU.
†Prior responder to 5-FU infusion.

kg/day. A PR in liver size was determined by the method of Moertel et al (9): a decrease of at least 30% in the sum of the measurements during quiet respiration below the xiphoid process and both costal margins at the midclavicular lines. Progressive hepatic disease was defined as an increase of at least 30% utilizing the technique of Moertel (9). Since HAI with 5-FUDR may alter hepatic enzymes, these parameters were not considered in assessing the responsiveness in these patients. Serial liver scans were obtained and correlation with clinical findings was necessary for patients to be coded as responders. While the liver was being perfused, no additional systemic chemotherapy was utilized.

RESULTS

Eight of 21 patients (35%) responded to HAI with 5-FUDR. The remissions ranged from 2 to 9 months. The median and mean length of remission was 4.5 and 5.0 months respectively. The duration of each patient's response is shown in table 1.

The median and mean survival for responders from the start of HAI was 8.0 and 9.0 months respectively. The range of survival was 2–15 months. The survival of responders compared to nonresponders was statistically significant at $P < 0.01$.[6] The median and mean survival of the nonresponders from the start of HAI was 1.0 and 1.6 months respectively. In both the responding and nonresponding groups there was one patient (table 1) who had previously responded to systemic 5-FU but had evidence of progressive disease at the initiation of HAI. The survival of all treated patients is shown in table 1.

The performance status (PS) at the time of placement of the hepatic artery catheter for both responders and nonresponders ranged from 20% to 90%. For the responding group the median and

[6]*P* value determined by student's "t" test.

mean PSs were 65% and 64% respectively, while in the nonresponder group the median and mean PSs were 60% and 53% respectively. There was no statistical difference between the PSs of the responders and nonresponders when the hepatic artery catheter was placed ($P = 0.45$).[6] The median and mean duration of disease from diagnosis to initiation of HAI for the responders was 11.5 and 14.4 months respectively, with a range of 2–41 months. For the nonresponders the median and mean duration of disease was 12.0 and 20.3 months respectively, with a range of 1–84 months. Thus, these two groups are comparable with respect to the natural behavior of their disease.

No hepatotoxicity, gastrointestinal intolerance, or myelosuppression was noted with the HAI.

DISCUSSION

Reported response rates in the literature for HAI with 5-FUDR in patients with metastatic colon carcinoma vary from 40% to 70% (1–5). Ansfield et al (10,11) have reported on patients treated with 5-FU via hepatic artery catheter after having progressed on systemic 5-FU. Their reports differ from our current retrospective study in several respects. There was no mention of the PS with regard to the responder versus nonresponder group. There was no stratification of the organ involved with regard to previous systemic 5-FU. The HAI was for approximately 21 days only, with reinstitution at intermittent intervals, and 5-FU was used instead of 5-FUDR. Finally, after discontinuance of HAI, patients frequently were re-treated with systemic 5-FU.

Other authors have also reported on HAI in patients who had received prior systemic 5-FU (4,5). It is also difficult to ascertain from these reports whether a response to 5-FU systemically was relevant to HAI response. Our data suggest that we might expect a response in approximately one third of the patients who receive prior systemic 5-FU and fail.

Should we expect a higher response rate with HAI in those patients who have previously responded to systemic 5-FU as opposed to those who have failed? This question can not be answered from our data since only one patient in each group had responded to prior systemic 5-FU.

The response rate (35%) with a median and mean duration of response of 4.5 and 5.0 months respectively in this group of patients is thought to be significant. Possibly of more importance is the median survival of 8 months in responding patients, all but one of whom had already failed on systemic 5-FU. This is compared to Moertel's series of 299 5-FU failures whose median survival was only 2.4 months (12). However, since this group was not stratified according to extent of disease and PS, a strict comparison with our series is not possible.

In this series the PSs of both responders and nonresponders were not statistically different, thus, there was no bias favoring one group over the other. The poor overall survival of the nonresponders, who had PSs comparable to those of the responders prior to HAI therapy, re-enforces the aggressive behavior of their hepatic disease. The explanation proposed for the response to HAI after 5-FU failure is thought to be the ability to deliver a much higher tumoricidal concentration of 5-FUDR.

Since there is no currently available effective second-line chemotherapy for disseminated colon carcinoma, we feel a randomized prospective trial with HAI may be warranted to determine its effect on overall patient survival.

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Echocardiographic Evaluation of Adriamycin Cardiotoxicity in Children[1,2]

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SUMMARY

Thirty children with malignant solid tumors receiving adriamycin had serial evaluation of left ventricular (LV) function by echocardiography. The echocardiographic studies showed a progressive decrease in myocardial contractility as evidenced by changes in several parameters of LV function with increasing cumulative doses of the drug. Using the patients as their own controls, serial echocardiography appears to be a reliable method for detecting early alteration of cardiac function in the asymptomatic patient. This technique may prove useful to prevent cardiomyopathy secondary to adriamycin and to prolong therapy beyond the currently established limits.

[Cancer Treat Rep 60:1281–1284, 1976]

A severe dose-dependent cardiomyopathy is the major limiting factor in the prolonged use of adriamycin (1–11). It has been found to occur in 30% of the patients receiving a total cumulative dose of > 550 mg/m² body surface area (1).

Several studies have indicated that once clinical signs of congestive heart failure (CHF) appear there is a death rate of 30%–50% (1–4). As a result, the drug is usually discontinued when this total cumulative dose is reached (1,2). Early changes in cardiac performance in the asymptomatic patient are generally unrecognized. Electrocardiographic (ECG) changes have been noted to appear after drug administration (1–11). However, these changes are nonspecific and not dose related. A recent study in adults has found the systolic time intervals derived from the simultaneous recording of the ECG, phonocardiogram, and carotid pulse tracing to be valuable in detecting early signs of cardiotoxicity (12). Similarly, the use of serial determination of the ejection fraction by echocardiography has also been described (13).

It is the purpose of this study to assess the value of serial echocardiography in detecting early signs of adriamycin cardiotoxicity in children and to evaluate its use in predicting the appearance of CHF, as well as its possible role in determining the maximum dose of adriamycin for any given patient.

MATERIALS AND METHODS

Thirty children (9 months to 19 years of age) with malignant solid tumors were treated with adriamycin (50 mg/m²/dose) as part of the chemotherapy regimens at our institution; they received either monthly injections of adriamycin or two doses, 3 weeks apart, every 3 months. Three patients had received radiotherapy to the mediastinum and, therefore, were given adriamycin at a smaller dose of 40 mg/m²/dose.

A total of 136 echocardiograms were performed in the 30 patients. In 22 of them tracings were obtained before starting therapy. Generally, we attempted to obtain an echocardiogram before each dose of adriamycin was administered and occasionally more than one study was performed between doses. All echocardiograms were done with the patients in the supine position, using a Hoffrel ultrasonoscope interfaced with a Cambridge or Irex multichannel recorder as previously described (14). The

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[2]Adriamycin: NSC-123127; CAS reg. No. 23214-92-8.

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[4]We gratefully acknowledge the valuable assistance of Maurice Bubb, Data Analyst, Southwestern Ohio Regional Computer Center, for the statistical analysis of our data.

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transducer frequency was selected according to the patient's size and age and ranged between 2.25 and 5.0 MHz.

Four parameters of left ventricular (LV) function were evaluated: shortening fraction (SF), ejection fraction (EF), mean velocity of circumferential fiber shortening (VCF), and LV systolic time interval ratio (STI). The SF was determined by measuring the LV end-systolic (Bs) and -diastolic (Bd) dimensions (fig 1). In Bs the posterior ventricular wall and the interventricular septum approach each other as the LV contracts. In Bd the distance between the ventricular wall and the septum increases as the ventricle expands. The relationship between these two distances (Bd–Bs divided by Bd) represents the SF and provides a quantitative expression of ventricular contractility (14). In our laboratory, the normal SF varies from 28% to 38% (15). We believe that a > 5% decrease in the SF cannot be accounted for by differences in recording technique nor by physiologic variations and probably represents a significant change in myocardial performance.

The EF is a volumetric expression of the SF (14) and the mean VCF expresses ventricular contractility per unit of time (16). The STIs (pre-ejection period [PEP] and LV ejection time [LVET]), expressed as the ratio PEP/LVET, also provide information about myocardial function (17,18). Normally, the STI ratio varies from 0.28 to 0.38. In myocardial failure the PEP lengthens, the LVET shortens, and therefore, the PEP/LVET ratio increases above normal limits. Since the majority of the patients were under therapeutic protocols that required administration of adriamycin at varying intervals, and the influence of this variation upon myocardial function was largely unknown, the SF of seven patients who received the drug at regular intervals (4 weeks between doses) was also analyzed separately.

In addition to the echocardiograms, ECGs were obtained at the same time. None of the 30 children developed clinical signs of CHF during the study period.

RESULTS

In one of our patients a typical graph of serial determinations of the SF during a 1-year period (fig 2) showed a progressive decrease from a high normal level of 38% before initiating adriamycin therapy to an abnormally low level of 27% after 300 mg/m^2, and further decreased to 24% after 450 mg/m^2. The combined analysis of the 30 patients (fig 3) demonstrated a significant decrease in the SF with increasing cumulative doses ($r = -0.42$, $P = 0.0001$). The scatter of values observed may reflect the wide range of individual variation in a heterogeneous group of patients. The resulting regression line only shows the decreasing tendency of the SF and has no predictive value. Before the start of therapy only 5% of the patients (one of 22) had an abnormally low

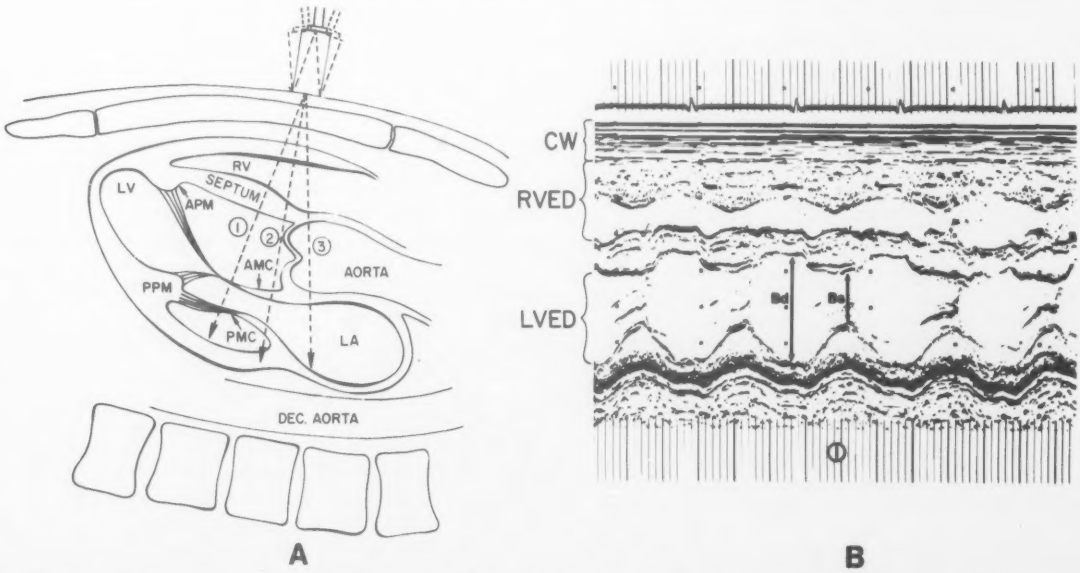

FIGURE 1.—This composite picture of (A) an idealized diagram of the heart in saggital view and (B) the corresponding echocardiogram shows the appropriate transducer position (No. 1) to record the LV dimensions. CW = chest wall; RVED = right ventricular end-diastolic dimension; LVED = LV end-diastolic dimension.

SF (< 28%), whereas 64% (nine of 14) of those who received 300 mg/m² had an SF < 28%.

The mean SF progressively decreased from a baseline value of 34.9% before therapy (a value similar to that of control patients in our laboratory) to 26.7% after 400 mg/m². Analysis of the SF from the small group of patients receiving the drug at regular intervals also showed a progressive decrease with increasing doses ($r = -0.55$, $P = 0.0007$) (fig 4). Similarly, increasing cumulative doses of adriamycin were associated with a decrease in the EF ($r = 0.40$, $P = 0.0001$), a decrease in the mean VCF ($r = -0.35$, $P = 0.0001$), and an increase in the STI ratio ($r = 0.17$, $P = 0.05$).

An analysis of the ECGs revealed abnormalities in 10%. They consisted of premature atrial contractions, premature ventricular contractions, and non-specific ST-T wave changes which were not dose related.

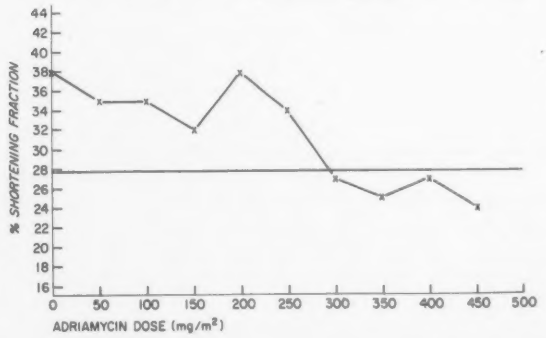

FIGURE 2.—Serial determination of the SF in a patient being treated with adriamycin for 1 year shows a progressive decrease from a high normal level of 38% before therapy to an abnormally low level of 24% after 450 mg/m². The horizontal line represents the lower limit of normal.

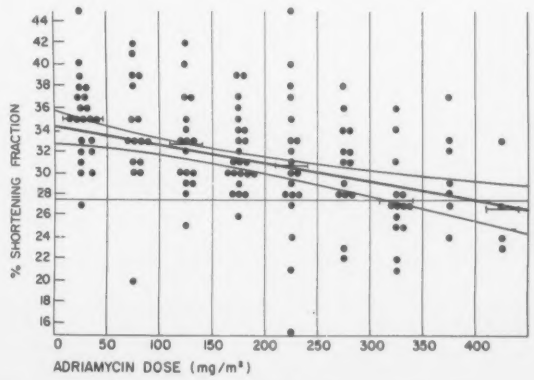

FIGURE 3.—Regression line and 95% confidence limits of the mean obtained from analysis of 30 patients showing a progressive decrease in the % SF with increasing doses of adriamycin. The horizontal bars represent the mean % SF for the corresponding dose level.

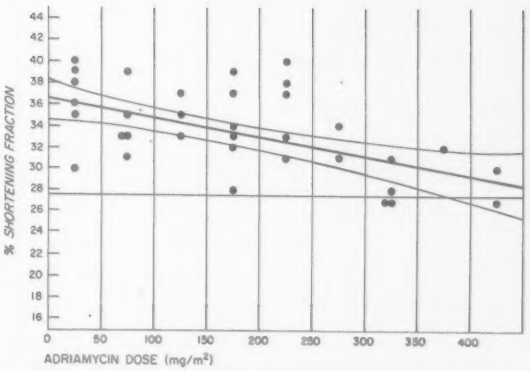

FIGURE 4.—Regression line and 95% confidence limits of the mean obtained from 7 patients who received adriamycin at regular 1-month intervals showing a progressive decrease in the % SF with increasing doses of adriamycin.

DISCUSSION

The identification of patients with a risk of developing cardiomyopathy secondary to adriamycin has been very difficult. Even the earliest clinical and radiologic changes are too late to prevent CHF, and immediate cessation of therapy is still associated with a significant number of deaths.

At the present time it appears that echocardiography is the best noninvasive method available to assess myocardial function. Minimal changes in LV function can be accurately monitored and consistently reproduced.

The data presented here indicate that the cardiotoxic effects of adriamycin may be recognized by echocardiography before clinical signs of CHF appear and, in fact, shortly after initiation of therapy. These effects consist of a dose-related progressive decrease in myocardial contractility as evidenced by changes in the SF, EF, mean VCF, and STI ratio.

After this preliminary study we conclude that: (*a*) using the patients as their own controls, serial echocardiography is a sensitive method to detect early alterations of myocardial function in the asymptomatic child receiving adriamycin; and (*b*) the simplest and probably the best parameter of LV function to be monitored in these patients is the SF.

It is hoped that further study at higher dose levels will identify those patients with a risk of developing CHF. We speculate that by stopping the drug at the appropriate time in the patients at risk, it may be possible to prevent CHF. In addition, it

may be possible to prolong therapy beyond the currently established limit in the presence of adequate cardiac function.

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Studies of the Effects of Cyclophosphamide, Vincristine, and Prednisone on Some Hepatic Oxidations and Conjugations[1,2,3]

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SUMMARY

The effects of low and high doses of three anticancer agents, cyclophosphamide, vincristine, and prednisone (given individually or in various combinations), on oxidative and conjugation pathways were studied in Sprague-Dawley male rats. Cyclophosphamide used alone at low doses decreased aniline hydroxylase and ethylmorphine demethylase activities by about 20% and at high doses produced a 30%–50% decrease in the specific activities of several microsomal mixed-function oxygenase activities, in the contents of cytochromes P-450 and b_5, and in the magnitudes of type I and II drug-binding spectrum. The levels of microsomal glucuronidase, glucuronyl transferase, and sulfatase per gram of liver were also decreased (30%–50%) by the high dose of cyclophosphamide. The high dose of cyclophosphamide in conjunction with either vincristine or prednisone also produced a noticeable decrease in several activities tested; however, when cyclophosphamide was given at either low or high doses in combination with vincristine and prednisone, the activities tested were comparable to those seen in untreated controls. The mechanism of this protection is presently unknown. Vincristine, at both low and high doses, produced little effect on oxidative pathways; however, at low doses it caused a significant increase (80%) in the specific activity of hepatic microsomal sulfatase. This effect was also discernible when vincristine was given in combination with cyclophosphamide and prednisone. Other than producing a 15% decrease in liver weight and a 40% decrease in the specific activity of microsomal glucuronidase, the high dose of prednisone used had no effect on various activities tested. Results of these studies indicate a potential for drug interaction among anticancer agents and supportive drugs used in combination cancer chemotherapy.

[Cancer Treat Rep 60:1285–1294, 1976]

Three sets of enzymes are involved in the biotransformation of drugs, carcinogens, and various other xenobiotics. The first set of enzymes, microsomal mixed-function oxygenase (MFO), carry out mainly oxidative reactions, introducing into the molecule polar functions which subsequently are conjugated with endogenous molecules by the second set of enzymes. A third set of enzymes, hydrolytic in function, can modify the end product of the second set of reactions, and in some cases even

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[3]Cyclophosphamide: NSC-26271; CAS reg. No. 6055-19-2; 2*H*-1,3,2-oxazaphosphorine, 2-[bis(2-chloroethyl)amino]tetrahydro-, 2-oxide, monohydrate.

Vincristine: NSC-67574; CAS reg. No. 2068-78-2; leurocristine, sulfate (1:1).

Prednisone: NSC-10023; CAS reg. No. 53-03-2; pregna-1,4-diene-3,11,20-trione, 17,21-dihydroxy-.

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modify the initial drug molecule, eg, hydrolysis of acetylsalicylic acid (1,2). During the course of therapy it is not uncommon for a cancer patient to receive a multitude of drug combinations including not only the anticancer agents but also several supportive drugs. Most of these supportive drugs and some anticancer drugs, eg, cyclophosphamide, isophosphamide, procarbazine, and aniline mustard, are activated and/or deactivated by the concerted and sequential action of MFO, conjugating, and hydrolytic enzymes (3–6). Although a number of anticancer drugs (cyclophosphamide, 5-fluorouracil, methotrexate, and 6-mercaptopurine) are known to depress MFO activities (7,8), their effects on the metabolism of other drugs and on various conjugation and hydrolytic pathways remain largely unknown at present. Since such information may be important in relation to cancer therapy, we studied the effects of cyclophosphamide (C), vincristine (O), and prednisone (P), given alone or in various combinations to rats, on the in vitro activities of various enzymes representative of oxidative, conjugative, and hydrolytic pathways. The COP combination was selected because: (*a*) cyclophosphamide is activated by hepatic MFO and also depresses the same enzyme system (7,9); (*b*) information concerning the effects of vincristine on various drug biotransformation pathways may have some potential therapeutic value; and (*c*) the COP combination has been used in the treatment of leukemia, Hodgkin's disease, and other forms of cancer (10). The enzyme activities specifically studied included demethylations of aminopyrine, ethylmorphine, propoxyphene (Darvon), meperidine (Demerol), atropine, and scopolamine, and hydroxylation of benzo(*a*)pyrene (BP) and aniline. Other measurements included levels of cytochromes P-450 and b_5; magnitudes of type I and II drug-binding spectrum; and glucuronyl transferase, glucuronidase, and sulfatase activities. Some of the selected drugs, which were used as in vitro substrates for MFO metabolism in this study, are used in the supportive care of cancer patients. The results of these studies are presented in this paper.

MATERIALS AND METHODS

Chemicals

Various drugs (cyclophosphamide, vincristine, prednisone, propoxyphene, meperidine, atropine, scopolamine, and ethylmorphine) were obtained from the pharmacy of this Institute. Aminopyrine and BP were the products of Aldrich Chemical Co, Milwaukee, Wis. BP was purified by repeated recrystallization from benzene. *p*-Nitro[2,6-^{14}C] phenol of 99% purity was obtained from ICN Chemical and Radioisotope Division, Irvine, Calif; *p*-nitrophenol, *p*-nitrophenylglucuronide, *p*-nitrophenyl sulfate, uridine diphosphoglucuronic acid, NADP, DL-isocitrate, and isocitrate dehydrogenase (type IV) were obtained from Sigma Chemical Co, St. Louis, Mo.

Animal and Tissue Preparation

Male Sprague-Dawley rats (200–300 g) received food and water ad libitum until the day of sacrifice. Rats received either one of the three drugs (cyclophosphamide, vincristine, or prednisone) or a combination of two or three of these drugs. Two dose regimens, low and high, were employed. The low-dose regimen consisted of the following treatment: cyclophosphamide, 50 mg/kg ip in saline on Day 1; vincristine, 0.05 mg/kg ip or iv in saline also on Day 1; prednisone, 4 mg/kg ip as a suspension in saline on Days 1–5; COP on Day 1 and prednisone alone on Days 2–5. Controls received an equivalent volume of saline only. The high-dose regimen was as follows: cyclophosphamide, 180 mg/kg ip on Day 1; vincristine, 0.28 mg/kg ip or iv on Day 1; prednisone, 14.3 mg/kg ip on Days 1–5; COP in the respective doses described above; controls, saline only. Cyclophosphamide was given on one side of the peritoneal cavity, followed ½ hour later with vincristine on the other side and ½ hour later with prednisone on the side on which cyclophosphamide was given. Alternatively, in order to avoid any possibilities of the drugs interfering with the absorption of each other in the peritoneal cavity, vincristine was given iv by tail vein and it was determined by such studies that the protocol for giving the COP combination did not produce interference with drug absorption since similar results were obtained whether vincristine was given iv or ip, preceded by cyclophosphamide and followed by prednisone.

In each of the three low-dose experiments, 15 rats were assigned randomly to five groups, three rats per group. Each group received one of the following treatments: cyclophosphamide, vincristine, prednisone, COP, or saline alone. For high-dose studies, two series of experiments were carried out. In one series 15 rats were assigned randomly to five groups, three rats per group, and given the following treatments: cyclophosphamide, vincristine, prednisone, COP, or saline only. This series was repeated three times. In another series of experiments 15 rats were assigned randomly to five groups (three rats per group) and given the following treatments: cyclophosphamide, cyclophosphamide and vincristine, cyclophosphamide and prednisone, COP, or saline only; this series was repeated twice. This experimental protocol ensured proper comparisons be-

tween various treatments applied simultaneously. Because of the number of rats used and the number of assays to be conducted on the same day, studies in each experiment were limited to five treatment groups only (including control). In low-dose experiments only a limited number of MFO activities were studied, but in high-dose studies many more activities were analyzed.

Animals were killed by decapitation on Day 8 between 8 and 9 AM. Livers were excised, perfused with cold isotonic saline, pooled from three rats of each group, and homogenized in cold sucrose solution (0.25 M) containing 1 mM EDTA, in a ratio of 1 g liver to 3 ml of sucrose solution. Homogenization was carried out in a glass homogenizing tube fitted with a teflon-coated pestle. The homogenate was centrifuged at 15,000 × *g* for 15 minutes and the resulting supernatant was centrifuged in several tubes containing equal volumes at 105,000 × *g* for 90 minutes in a Beckman Model L5-50 ultracentrifuge to prepare the microsomal pellets, which were then suspended in potassium phosphate buffer (0.1 M) (pH 7.4) or in 0.15 KCl and homogenized lightly. In some experiments crude lysosomal fraction was also prepared by centrifuging the homogenate first at 600 *g* for 20 minutes, then at 15,000 *g* for 15 minutes; the 15,000-*g* pellet was homogenized in 0.15 M KCl. All cell fractionation procedures were carried out at 0°–4°.

Studies on various MFO activities were carried out with the microsomal suspensions prepared in phosphate buffer, whereas studies on other activities, conjugation and hydrolysis, were carried out using microsomal suspensions prepared in 0.15 M KCl. In experiments where other liver subfractions or homogenates were used, respective suspensions in EDTA-sucrose solution were diluted threefold with phosphate or acetate buffer. All MFO activities, including determinations of cytochromes P-450 and b_5 and type I and II drug-binding spectrum, were assayed in potassium phosphate (pH 7.4) buffer, and other activities reported in this paper were assayed in citrate buffer unless stated otherwise.

Two sets of blanks were run for MFO activities: one in which the incubation mixture contained everything except the NADPH-generating system and was treated in the same way as the experimental incubation, and another in which microsomes were added after the reaction was stopped. In both cases blank values were low.

The concentration of the drug substrate in the incubation mixture was not limiting because the saturating concentration was used in each case; this concentration was determined in preliminary experiments and was found to be 8 mM for all substrates undergoing demethylation except aminopyrine for which it was 6 mM. For aniline this concentration was 8 mM, and for BP it was 0.1 mM. These substrate concentrations were selected in the plateau region of velocity versus substrate plots. The concentration of the microsomal protein in the incubation mixture ranged from 1.5 to 2 mg/ml and the reaction was linear with respect to the times of incubation which were as follows: aminopyrine and meperidine, 5 minutes; propoxyphene, ethylmorphine, atropine, and scopolamine, 10 minutes; aniline, 15 minutes; and BP, 15 minutes.

Determination of Drug Substrate Demethylation

The standard 3-ml incubation mixture contained 0.07 M KPO_4 buffer (pH 7.4); $MgCl_2$, 5 mM; $MnCl_2$, 1 mM; an NADPH-generating system consisting of NADP, 0.33 mM, DL-isocitrate, 8 mM; and Sigma type IV isocitrate dehydrogenase (10 μg protein/ml) and the respective drug substrate. This buffer mixture (minus substrate) was prewarmed at 37°C for 10 minutes to generate an adequate amount of NADPH needed for the reaction which was started with the addition of microsomes. Formaldehyde resulting from the oxidation of the drug substrate was quantitated by reaction with the Nash reagent as described by Schenkman et al (11).

Determination of Aniline Hydroxylation

The composition of the incubation mixture was the same as described above, except that the concentration of the substrate aniline was 8 mM. The reaction was terminated with the addition of 1 ml of 20% trichloroacetic acid and assayed for *p*-aminophenol as described by Schenkman et al (11).

Determination of BP Hydroxylation

The reaction was carried out in 1 ml of the incubation mixture, using 100 μg of microsomal protein and 0.1 mM BP. The reaction was assayed essentially according to the method of Nebert and Gelboin (12). This involved extraction of the reaction mixture with a solvent mixture of hexane:acetone (3:1), which was separated from the aqueous phase and re-extracted with 1 N NaOH. The alkali extracted the phenols which were quantitated in terms of relative intensity of their fluorescence, measured in an Aminco-Bowman fluorometer using an excitation wavelength of 396 nm and an emission wavelength of 522 nm.

Determination of Microsomal Cytochromes

Cytochromes P-450 and b_5 were determined by the previously described procedures (13). A solution of microsomal protein (1.5 to 2 mg/ml) was equally divided between the two cuvettes and the base line of equal light absorbance was recorded. Then the contents of sample cuvette were reduced with dithionite and the spectrum between 390 and 500 nm was recorded and concentration of cytochrome b_5 was calculated using a millimolar extinction coefficient of 171 (13). For the cytochrome P-450 measurement, the microsomal solution was equally divided between the two cuvettes and the base line of equal absorbance was recorded, dithionite was added to both cuvettes and mixed well, and then CO was bubbled for 30 seconds through the contents of the sample cuvette only and the spectrum was recorded. A millimolar extinction coefficient of 91 was used to calculate the concentration of cytochrome P-450 (13). All spectra were recorded with an Aminco DW-2 recording spectrophotometer.

Determination of Drug-Binding Spectrum

Microsomal solution (2 mg/ml) was equally divided between the two cuvettes and the base line of equal light absorbance was recorded. Then either type I (hexobarbital to a final concentration of 5 mM) or type II (aniline to a final concentration of 3 mM) substrate was added in a small volume to the sample cuvette only and mixed well with the microsomal suspension, and the spectrum was recorded between 350 and 500 nm. Type I drug-binding spectrum has a peak between 385 and 392 nm and a trough between 420 and 425 nm; type II spectrum has a peak between 427 and 432 nm and a trough between 390 and 395 nm (11). These spectra were obtained on an Aminco DW-2 recording spectrophotometer.

Determination of Glucuronyl Transferase Activity

This activity was determined using 0.5 mM ^{14}C-*p*-nitrophenol, 2 mM uridine diphosphoglucuronic acid, 0.2 M phosphate buffer at pH 7.4, and homogenate or microsomes equivalent to 0.1 g liver/ml of incubation medium. The radioactive *p*-nitrophenylglucuronide was determined by the method of Gessner (14).

Determination of Glucuronidase Activity

Homogenates or microsomes (equivalent to 0.1 g of tissue/ml of incubation medium) were incubated with 1 mM *p*-nitrophenylglucuronide in 0.1 M acetate buffer at pH 5. The incubations were carried out for 15 minutes at 37°C and were stopped by the addition of 3 volumes of ethanol followed by freezing. Ethanolic mixtures were centrifuged and aliquots of the supernatants were diluted with 3–9 volumes of 1 N NaOH. Optical density readings of the alkaline supernatants were taken at 400 nm. The amount of the product (*p*-nitrophenol) formed was deduced from appropriate calibration curves and by subtracting readings of 0 time incubations treated analogously to the 15-minute incubations.

Determination of Sulfatase Activity

Homogenate, lysosomal, or microsomal preparations (equivalent to 0.1 g of tissue/ml of incubation medium) were incubated with 1 mM *p*-nitrophenylsulfate in 0.1 M acetate buffer at pH 5.0 for lysosomal sulfatase activity, and in 0.1 M tris-malate buffer at pH 7.6 for microsomal sulfatase activity. The incubations were carried out for 15 minutes at 37°C and were stopped by the addition of 3 volumes of ethanol followed by freezing. The amount of *p*-nitrophenol formed was analyzed in the manner described above for glucuronidase activity.

RESULTS

Effects of Cyclophosphamide Alone or in Combination

A single dose of cyclophosphamide (50 mg/kg ip) administered to rats 1 week before assays of the enumerated hepatic enzymes resulted in a statistically significant decrease (about 20%) in both ethylmorphine demethylase and aniline hydroxylase activities (tables 1 and 2). A depressed activity of ethylmorphine demethylase was also discernible in the COP combination group (table 1).

When cyclophosphamide was administered at the higher dose level, 180 mg/kg ip, striking effects were observed on almost all the hepatic parameters examined. Thus, there was a significant decrease (about 30%) in hepatic microsomal protein and an even greater decrease (about 50%) in the cytochrome P-450 content of microsomes; there was also a lesser but significant decrease (about 30%) in the microsomal cytochrome b_5 level (table 3). Concomitant with these decreases, specific activities of the cytochrome-dependent demethylations and hydroxylations were depressed by 30%–50% (tables 2 and 4), except for atropine demethylation, which was not significantly affected. Type I and II interactions (*see* Methods) with cytochrome P-450 were also depressed by cyclophosphamide given alone or in combination with vincristine or prednisone; these effects approximately paralleled the levels of cyto-

TABLE 1.—Comparison of effects of low-dose treatment on rat liver weights, yields of microsomal protein, levels of microsomal cytochromes P-450 and b_5, and microsomal demethylations*

Treatment	Liver weight	Protein yield	Cytochrome		Amino-pyrine	Ethyl-morphine
			P-450	b_5		
Cyclophosphamide	85 ± 7 (3)	108 ± 5 (3)	87 ± 5 (3)	99 ± 5 (3)	90 ± 12 (3)	78 ± 2 (3)†
Vincristine	95 ± 7 (3)	125 ± 13 (3)	91 ± 2 (3)	95 ± 1 (3)	92 ± 11 (3)	76 ± 9 (3)
Prednisone	92 ± 7 (3)	113 ± 11 (3)	107 ± 12 (3)	109 ± 7 (3)	116 ± 29 (3)	81 ± 4 (3)†
COP	88 ± 1 (3)†	97 ± 3 (3)	111 ± 22 (3)	108 ± 12 (3)	112 ± 24 (3)	90 ± 2 (3)†

*Controls = 100, values are % of respective controls; control values as mean ± SE (No. of experiments) were as follows: liver weight in g/rat, 12.5 ± 0.4(3); microsomal protein in mg/g of liver,14.0 ± 2(3); cytochrome P-450 in nmols/mg of microsomal protein, 0.97 ± 0.2(3); cytochrome b_5 in nmols/mg of microsomal protein, 0.30 ± 0.04(3); aminopyrine demethylase in nmols of HCHO formed/mg of microsomal protein/hr, 340 ± 56(3); ethylmorphine demethylase in nmols of HCHO formed/mg of microsomal protein/hr, 284 ± 36(3). Blank values in terms of absorbance at 412 nm ranged between 0.025 and 0.035 compared to the control values ranging from 0.10 to 0.5 for various demethylation reactions reported in this and other tables. Blank for aryl hydrocarbon hydroxylase assay was < 0.2% of control value.

†$P < 0.01$.

TABLE 2.—Comparison of effects of various treatments on specific activities of microsomal hydroxylases and magnitude of interactions of cytochrome P-450 with a type I substrate (hexobarbital) and a type II substrate (aniline)*

Treatment	Hydroxylations		P-450 interactions	
	Aniline	BP	Type I	Type II
High dose:				
Cyclophosphamide	57 ± 8 (5)†	61 ± 11 (4)‡	56 ± 13 (4)‡	54 ± 13 (4)‡
Cyclophosphamide + vincristine	44 ± 39 (2)	47 ± 16 (2)	56 ± 16 (2)	47 ± 22 (2)
Cyclophosphamide + prednisone	36 ± 18 (2)	57 ± 10 (2)	47 ± 8 (2)	40 ± 3 (2)
Vincristine	112 ± 3 (3)	109 ± 8 (2)	100 ± 2 (2)	95 ± 8 (2)
Prednisone	102 ± 9 (3)	117 ± 18 (2)	122 ± 13 (2)	119 ± 14 (2)
COP	93 ± 16 (5)	80 ± 16 (4)	80 ± 7 (4)	77 ± 13 (4)
Low dose:				
Cyclophosphamide	88 ± 2 (3)†	78 ± 6 (2)	107 ± 16 (2)	95 ± 5 (2)
Vincristine	86 ± 9 (3)	91 ± 12 (2)	102 ± 6 (2)	96 ± 4 (2)
Prednisone	101 ± 16 (3)	97 ± 4 (2)	116 ± 6 (2)	116 ± 16 (2)
COP	116 ± 22 (3)	89 ± 4 (2)	106 ± 25 (2)	126 ± 37 (2)

*Control = 100, values are % of respective controls; control activities as mean ± SE (No. of experiments) were as follows. *High dose:* aniline hydroxylase activity in nmols of *p*-aminophenol formed/mg of microsomal protejn/hr, 24 ± 2(5); BP hydroxylase activity as relative intensity units due to the formation of phenols/mg of microsomal protein/15 mins, 30 ± 2(4); drug-binding spectrum type I as change in optical density, 391–421 nm × 10^{-3}, 25 ± 3(4); type II as change in optical density, 428–395 nm × 10^{-3}, 30 ± 2(4). *Low dose* (same units as for high-dose experiments): aniline hydroxylase, 35 ± 9(3); BP hydroxylase, 29 ± 1(2); type I drug-binding spectrum, 17 ± 8(2); type II drug-binding spectrum, 28 ± 8(2).

†$P < 0.01$.

‡$P < 0.05$.

TABLE 3.—Comparison of effects of high-dose treatment on rat liver weights, yields of microsomal protein, and microsomal cytochrome P-450 and b_5 levels*

Treatment	Liver weight	Protein yield	Cytochrome	
			P-450	b_5
Cyclophosphamide	90 ± 7 (5)	71 ± 9 (5)†	52 ± 9 (5)†	73 ± 7 (5)†
Cyclophosphamide + vincristine	87 ± 18 (2)	90 ± 4 (2)	59 ± 19 (2)	33 ± 1 (2)
Cyclophosphamide + prednisone	80 ± 9 (2)	88 ± 3 (2)	48 ± 4 (2)	77 ± 5 (2)
Vincristine	89 ± 13 (3)	75 ± 20 (3)	91 ± 1 (3)	97 ± 3 (3)
Prednisone	84 ± 5 (3)‡	73 ± 14 (3)	95 ± 18 (3)	97 ± 16 (3)
COP	82 ± 3 (5)†	83 ± 11 (5)	77 ± 11 (5)	99 ± 4 (5)

*Controls = 100, values are % of respective controls; control values as mean ± SE (No. of experiments) were as follows: liver weight in g/rat, 11.1 ± 0.6(5); microsomal protein in mg/g of liver, 15.8 ± 2.2(5); cytochrome P-450 in nmols/mg of protein, 0.97 ± 0.05(5); cytochrome b_5 in nmols/mg of microsomal protein, 0.34 ± 0.02(5).

†$P < 0.01$.

‡$P < 0.05$.

chrome P-450 in the microsomal preparations of the various groups (compare tables 3 and 4). Hepatic microsomal glucuronidase, glucuronyl transferase, and sulfatase per gram of liver were also significantly depressed by about 30%–50%; this effect was chiefly attributable to a decreased level of hepatic microsomal protein, since the specific activities of the latter three enzymes were not significantly affected (table 5). This is in contrast to the pronounced decreases in specific activities of mixed-function oxidases (tables 2 and 4).

The high-dose treatment with cyclophosphamide in combination with vincristine or prednisone also tended to depress cytochromes P-450 and b_5 and mixed-function oxidases (tables 3 and 4).

Effects of Vincristine

Treatment of rats with a single low dose of vincristine, 0.05 mg/kg, resulted 1 week later in about an 80% increase in the specific activity of hepatic microsomal sulfatase (table 6) and this effect appeared to be unique among enzyme activities tested.

The higher dose of vincristine, 0.28 mg/kg, produced no significant effects on any of the mixed-function oxidases tested but tended to decrease the content of hepatic microsomal glucuronyl transferase. High-dose vincristine treatment increased markedly, by 65%, glucuronidase activity in liver homogenates but not in microsomes, suggesting that lysosomal glucuronidase might have been responsible for the increase (table 5).

Effects of Prednisone

On the eighth day following treatment with prednisone, 4 mg/kg for the first 5 days, hepatic microsomal ethylmorphine demethylase activity decreased by about 20% (table 1) while hepatic sulfatase levels tended to increase (table 6).

Treatment with a higher dose of prednisone, 14.3 mg/kg, produced about a 15% decrease in liver weights and about a 40% decrease in the specific activity of microsomal glucuronidase (tables 3 and 5).

TABLE 4.—Comparison of effects of high-dose treatment on specific activities of microsomal demethylases*

Treatment	Aminopyrine	Propoxyphene	Meperidine	Ethylmorphine	Atropine	Scopolamine
Cyclophosphamide	49 ± 8 (5)†	67 ± 14 (5)‡	70 ± 10 (4)‡	72 ± 12 (5)‡	80 ± 19 (5)	64 ± 9 (5)†
Cyclophosphamide + vincristine	52 ± 16 (2)	62 ± 54 (2)	83 (1)	57 ± 33 (2)	86 ± 62 (2)	54 ± 33 (2)
Cyclophosphamide + prednisone	43 ± 3 (2)	37 ± 15 (2)	43 (1)	38 ± 6 (2)	56 ± 26 (2)	40 ± 11 (2)
Vincristine	91 ± 4 (3)	134 ± 27 (3)	105 ± 22 (3)	128 ± 27 (3)	119 ± 2 (3)	123 ± 24 (3)
Prednisone	105 ± 14 (3)	95 ± 15 (3)	103 ± 14 (3)	109 ± 10 (3)	101 ± 2 (3)	109 ± 10 (3)
COP	90 ± 8 (5)	83 ± 12 (5)	83 ± 14 (5)	86 ± 15 (5)	92 ± 8 (5)	84 ± 10 (5)

*Controls = 100, values are % of respective controls; control values for *N*-demethylase activity/mg of microsomal protein/hr as mean ± SE (No. of experiments) were as follows: aminopyrine, 572 ± 89(5); propoxyphene, 85 ± 16(5); meperidine, 239 ± 57(4); ethylmorphine, 260 ± 51(5), atropine, 46 ± 10(5); scopolamine, 154 ± 7(5).

†$P < 0.01$.

‡$P < 0.05$.

TABLE 5.—Effect of COP treatment (high dose) on liver enzyme activities (calculated per g of tissue per min or as specific activities) compared to their respective control groups*

	Glucuronidase activity			Glucuronyl transferase activity			Microsomal sulfatase activity		
Treatment	Homogenate	Microsomal	Microsomal specific activity	Homogenate	Microsomal	Microsomal specific activity	Homogenate	Microsomal	Microsomal specific activity
Cyclophosphamide	92 ± 7 (3)	46 ± 8 (4)†	76 ± 15 (4)	87 ± 10 (3)	70 ± 20 (5)†	96 ± 7 (5)	102 ± 16 (3)	55 ± 5 (4)†	88 ± 4 (5)
Cyclophosphamide + vincristine	97 ± 13 (2)	63 ± 13 (2)	65 ± 14 (2)	105 ± 16 (2)	101 ± 4 (2)	114 ± 23 (2)	125 ± 41 (2)	114 ± 5 (2)	114 ± 4 (2)
Cyclophosphamide + prednisone	101 ± 18 (2)	50 ± 4 (2)	57 ± 6 (2)	97 ± 28 (2)	79 ± 13 (2)	84 ± 8 (2)	122 ± 22 (2)	105 ± 9 (2)	119 ± 15 (2)
Vincristine	165 ± 15 (2)	100 ± 5 (2)	107 ± 19 (2)	96 ± 12 (2)	78 ± 4 (2)	114 ± 14 (2)	86 ± 2 (2)	91 ± 6 (2)	100 ± 9 (2)
Prednisone	128 ± 24 (2)	92 ± 44 (2)	66 ± 6 (2)	88 ± 16 (2)	78 ± 7 (2)	84 ± 5 (2)	108 ± 1 (2)	88 ± 7 (2)	90 ± 10 (3)
COP	112 ± 16 (4)	60 ± 13 (4)	65 ± 12 (4)	100 ± 15 (4)	103 ± 7 (3)	92 ± 12 (5)	122 ± 17 (4)	110 ± 6 (4)	116 ± 6 (5)

*Controls = 100; values are % of respective controls; control activities in nmols/g of liver/min ranged as follows: glucuronidase—homogenate, 214–402, and microsomal, 72–120; glucuronyl transferase—homogenate, 40–53, and microsomal, 11–15; sulfatase—homogenate, 117–175, and microsomal, 31–54. Control specific activities of microsomal preparations in nmols/mg of protein/min ranged as follows: glucuronidase, 4.5–11.6; glucuronyl transferase 1.0–1.7; sulfatase, 2.1–4.9.

†$P < 0.01$.

TABLE 6.—Effect of COP treatment (low dose) on liver enzyme activities (calculated per g of tissue per min) compared to their respective control groups*

	Glucuronidase			Glucuronyl transferase		Microsomal sulfatase		Lysosomal sulfatase	
Treatment	Homogenate	Lysosomal	Microsomal	Homogenate	Microsomal	Homogenate	Microsomal	Homogenate	Lysosomal
Cyclophosphamide	112 ± 2 (2)	116 ± 32 (2)	88 ± 15 (3)	95 ± 7 (2)	133 ± 10 (3)	114 ± 20 (2)	138 ± 23 (3)	119 ± 62 (2)	93 ± 32 (2)
Vincristine	109 ± 6 (2)	102 ± 32 (2)	117 ± 16 (3)	87 ± 15 (2)	110 ± 1 (3)	135 ± 26 (2)	178 ± 12 (3)†	134 ± 44 (2)	139 ± 15 (2)
Prednisone	97 ± 11 (2)	115 ± 12 (2)	106 ± 4 (3)	91 ± 18 (2)	108 ± 5 (2)	121 ± 5 (2)	154 ± 31 (3)	119 ± 24 (2)	126 ± 24 (3)
COP	86 ± 5 (2)	101 ± 29 (2)	94 ± 17 (3)	88 ± 12 (2)	92 ± 4 (3)	128 ± 1 (2)	141 ± 14 (3)‡	121 ± 13 (2)	149 ± 2 (2)

*Controls = 100, values are % of respective controls; control enzyme activities in nmols/g of liver/min ranged as follows: glucuronidase—homogenate, 215–261, lysosomal, 67–76, and microsomal, 71–92; glucuronyl transferase—homogenate, 40–53, and microsomal, 11–14; microsomal sulfatase—homogenate, 112–146, and microsomal, 30–40; lysosomal sulfatase—homogenate, 31–36, and lysosomal, 6–7.

†$P < 0.01$.

‡$P < 0.05$.

Effects of COP Combination Treatment

The combination treatment, both at low and high dose levels, produced about a 15% decrease in liver weights (tables 1 and 3). Low-dose COP treatment caused about a 50% increase in both microsomal and lysosomal sulfatases (table 6); this effect was attributable chiefly to the vincristine component of the regimen.

High-dose COP treatment generally produced an insignificant depression of the various parameters tested, except for a decrease in liver weight, in spite of the striking effects observed when cyclophosphamide was used alone.

DISCUSSION

Male Sprague-Dawley rats were treated with either a low-dose or a high-dose regimen of COP, each drug given alone or in combination with the other drugs in a protocol involving a single injection of cyclophosphamide and vincristine and multiple injections of prednisone, and 7 days later various activities (representative of drug oxidative, conjugative, and hydrolytic pathways) were examined in the microsomes or various other hepatocellular subfractions. All the data in this paper have been calculated as percent increases or decreases relative to controls, carried out contemporaneously. In this way variations observed in various experiments, possibly related to different batches of rats used, have been normalized. Variations in the activities of microsomal oxygenase, caused by the differences in the batches of rats, are not uncommon; it has been reported previously that during a period of 2.5 years the cytochrome P-450 (terminal enzyme in the microsomal oxygenase complex) content varied by > 300% (15).

The low dose of cyclophosphamide corresponded to the therapeutic dose for rat Dunning leukemia IRC-741 (16) and the high dose was in the range of that used by other investigators for studies of effects of cyclophosphamide on drug metabolism (3,17). The results reported here show that cyclophosphamide treatment produces a specific effect in depressing microsomal mixed-function oxidations, that ethylmorphine demethylation is especially sensitive to these effects, and that at higher doses cyclophosphamide specifically lowers the cytochrome P-450 content of microsomes and the cytochrome b_5 content to a lesser degree. Since metabolic activation of cyclophosphamide to alkylating species (18) is a cytochrome P-450-dependent reaction, it is possible that generation of the alkylating molecules in the proximity of the cytochrome results in a certain amount of in situ alkylation and denaturation of the cytochrome, leading to a lower microsomal content of the cytochrome. Subsequently, depressed protein synthesis may be responsible for the slow recovery and the long-term (7-day) effect. These effects of cyclophosphamide seem to be moderated by the other drugs of the COP regimen. The mechanism of this "protection" cannot be explained at this time.

Prednisone doses were chosen to be roughly equivalent to the doses used in man, taking into consideration that the metabolic rate of rats is ten times faster than that of man and that this steroid is extensively metabolized. The lower dose of prednisone caused a slight decrease in ethylmorphine demethylase. The higher dose treatment produced a small but significant decrease in liver weight and a marked decrease in specific activity of microsomal glucuronidase. The decrease in liver weight is probably due to the known effects of corticosteroids on protein catabolism.

The high dose of vincristine, 0.28 mg/kg, was close to the threshold of the neurotoxic dose for rats (19). In fact, in some of our animals we observed a transient limb paralysis. The lower dose of 0.05 mg/kg should have produced moderately cytotoxic concentrations (20), since at this dose the distribution of vincristine in body water should have given a concentration of about 1×10^{-7} M or 0.1 μg/ml. It is interesting that vincristine significantly increased the level of hepatic sulfatase at the lower dose and somewhat decreased it at the higher dose. At the higher dose, the effect was observable in the homogenate rather than in the microsomal fraction, suggesting that lysosomal sulfatase may have been affected. Glucuronidase, most likely lysosomal glucuronidase, was substantially increased by the high dose of vincristine. It remains to be explored whether the effects of vincristine on these membrane-bound enzymes are related to its disruptive effects on cell morphology (21). In any event these effects of vincristine on conjugation pathways, and especially on the lytic enzymes, suggest that vincristine treatment may have an effect on metabolism of drugs that need to be sulfated or glucuronidated for detoxication, and this represents a potential for drug interactions.

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Survival and Cycle-Progression Delay of Human Lymphoma Cells In Vitro Exposed to VP-16-213[1,2,3]

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SUMMARY

The lethal and kinetic effects of VP-16-213 were analyzed in a human lymphoid cell line (T_1 cells) in vitro. When asynchronous T_1 cells were exposed to increasing concentrations of VP-16-213 for 1 hour, an exponential survival curve with a D_0 (mean lethal dose equal to the concentration required to reduce survival by 63% on the exponential part of the survival curve) of 3 μg/ml was obtained. Increasing exposure time also reduced survival exponentially. Synchronized cells showed age-dependent sensitivity to VP-16-213 with the greatest lethal damage experienced by cells treated in S and G_2 phase. The major kinetic response of the T_1 cells to VP-16-213 was a delay in G_2 phase, the extent and duration of which was a function of drug concentration, exposure time, and cell cycle stage of drug addition; thus, cells in S phase were most effectively blocked in the subsequent G_2 phase. Continuous treatment with concentrations of VP-16-213 (> 0.5 μg/ml for > 3 hours) caused a retardation of S-phase transit with prompt recovery after drug release. Treatment with 10.0 μg/ml for > 3 hours resulted in a "frozen state" of the whole life cycle, inducing only minor compartment changes. DNA synthesis was inhibited in the majority of cells with an S-phase DNA content. There was no correlation between the extent of perturbation and lethal effects after treatment with VP-16-213.

[Cancer Treat Rep 60:1295–1306, 1976]

VP-16-213, a new anticancer drug presently undergoing clinical trials, is a semisynthetic derivative of podophyllotoxin closely related to another similar derivative, VM-26 (1–3). Both compounds have shown marked activity in experimental tumor systems and in human cancers, but the therapeutic index of VP-16-213 appears superior (2,4–7). While podophyllotoxin blocks the cell cycle progression in metaphase (8), VP-16-213 and VM-26 decrease the mitotic index (MI) and were presumed to arrest cells in the premitotic stage (1,2). This was later confirmed by the studies of Misra and Roberts (9) and Krishan et al (10). This study, on the effects of VP-16-213 on an in vitro human lymphoma cell line, reports on both lethal effects, as assessed by colony formation, and kinetic response, evaluated by means of pulse cytophotometry (PCP), as related to concentration, exposure time, and cell age. VP-16-213 is one of the most powerful cytotoxic agents thus far studied on this cell line. Cell killing is greatest in G_2 phase. We confirm the results of Grieder et al (1) in that delay of cell cycle progression occurs mainly in the G_2 phase. However, in contrast to the findings of Krishan et al (10), the transit through G_1 and S phase is retarded only with very high concentrations of VP-16-213 incubated for longer time periods.

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[3] VP-16-213: NSC-141540; 4′-demethylepipodophyllotoxin 9-(4,6-*O*-ethylidene-β-D-glucopyranoside). Supplied by Cancer Therapy Evaluation Program, DCT, NCI.

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MATERIALS AND METHODS

Drug

VP-16-213, manufactured by Sandoz Laboratories, Basel, Switzerland, was received in an organic solvent. Dilutions in growth medium were made fresh before each experiment.

Cell Line

A human immunoglobulin-producing cell line (T_1 cells) derived from the tumorous lymph node of a patient with lymphocytic lymphoma was used (11). Monolayer cultures were maintained in Ham's F-10 medium supplemented with 20% fetal calf serum, vitamins, glutamine, and antibiotics. Under these conditions, the cell cycle time recently determined by the pulse-labeled mitosis (PLM) method was 31 hours, the pre-DNA synthesis period (G_1) was 11 hours, the DNA synthesis period (S) was 10 hours, and the post-DNA synthesis period (G_2) was 10 hours.

Cell Survival

To assess survival, treated and control cells growing in 60-mm petri dishes were harvested by previously reported standard procedures (12) and counted with the aid of an electronic particle counter (Coulter Electronics, Inc, Hialeah, Fla). Known aliquots were dispensed into 60-mm petri dishes so that 50–100 colonies would appear after a 21-day incubation in a 5% CO_2-humidified atmosphere at 37°C. The colonies were stained with 0.5% crystal violet in 95% ethanol. Viability was defined as the ability of single cells to give rise to a colony of $\geq$ 50 cells. In each experiment, the plating efficiency of at least six controls was assessed simultaneously. The survival fractions for the different drug concentrations were normalized with respect to the individual controls for each experiment. In this series of experiments, the plating efficiency ranged from 15% to 40%. All experiments were done at least twice with three samples for each drug concentration and exposure time. Means, standard errors, and ancillary statistical analyses were calculated with the aid of a Cyber 73 computer (Control Data Corp, Minneapolis, Minn).

Cell Synchronization

Synchronized S-phase cells were obtained by a single 24-hour treatment with 3-mM thymidine (TdR). The degree of synchrony was monitored in replicate cultures by 30-minute pulse labeling with 1 microcurie of ^{3}H-cytidine/ml to determine the percentage of cells in S phase and by scoring the MI, and by DNA histograms obtained by PCP as previously described (13). In all radioautography procedures, a 50% solution of Ilford K5 emulsion (Polysciences, Inc, Warrington, Pa) in distilled water was used. Labeled cells were identified by the presence of $\geq$ 5 grains overlying the nucleus. At the end of the synchrony procedure, 90% of the cells were in early S phase and moved synchronously into G_2 (80%). After a mitotic peak of 5%–10% (the MI of asynchronous T_1 cells ranges from 0.6 to 1.8), the cells were still partially synchronized in G_1 (70%–80%). These procedures did not significantly alter the plating efficiency of T_1 cells.

Dose-Response Survival of Asynchronous Populations

Stock cultures of T_1 cells were harvested, and cell suspension aliquots were seeded into 60-mm petri dishes (0.5×10^6 cells/dish). The cells were incubated at 37°C in a 5% CO_2 atmosphere in air for about 48 hours to achieve exponential growth. The medium was discarded and the cells were exposed to increasing drug concentrations for exactly 1 hour at 37°C. The drug was decanted, the cells were washed twice with Hanks' balanced salt solution, and fresh medium was added. Colony-forming ability was assayed as described above.

Cell-Kill Kinetics

Cell survival as a function of duration of treatment was investigated by exposing replicate cultures simultaneously to a single dose of drug. Petri dishes containing 0.5×10^6 cells in asynchronous exponential growth were incubated at 0 time with a single concentration of drug (1 μg/ml). Replicate cultures were harvested at regular intervals for 48 hours and survival was assessed as described above. Plating efficiency controls were obtained at regular intervals. To demonstrate the stability of the biologic activity of the drug a separate control system was instituted. This consisted of dishes also containing 0.5×10^6 cells which were kept growing without adding drug throughout the experiment. Every time a time-point dish was harvested, its supernatant medium was transferred to the control dishes after discarding the drug-free medium. The latter was now incubated for 1 hour at 37°C and survival assessed as usual.

Cell Cycle-Dependent Cell Survival

Age-dependent survival was investigated by incubating synchronized cells with increasing concen-

trations of drug at regular intervals throughout the cell cycle. Plating efficiency controls were obtained at each time point.

Cell Cycle Progression

The effect of concentration and duration of treatment on cell cycle traverse was assessed by means of sequential DNA histograms. Asynchronous T_1 cells in exponential phase of growth were treated with 0.1, 0.5, 1.0, 5.0, and 10.0 μg/ml of VP-16-213 for various incubation times (1, 3, 12, and 48 hours). Replicate samples were harvested both during drug exposure and after drug removal following pulse-labeling with ^{3}H-TdR (1 microcurie/ml) for 30 minutes and were processed for radioautography and PCP. Two hundred cells were scored for labeling index (LI) determination and 3000 cells for MI. A total of 50,000–100,000 cells were measured for each DNA histogram. Age-dependent kinetic response was investigated by exposing TdR-synchronized cells to 5 and 10 μg/ml of VP-16-213 for 1 hour throughout the cell cycle. At 6–12-hour intervals treated and control samples were harvested and processed for radioautography and PCP. Evaluation of DNA histograms was performed as previously described (14). The standard deviation of the DNA histogram distribution did not exceed 1.5% for the $G_{1/0}$, S, and (G_2 + M) compartments.

RESULTS

Cell Survival

The survival curve of asynchronous exponentially growing T_1 cells exposed to increasing concentrations of VP-16-213 for 1 hour shows a straight logarithmic relation with no shoulder region (fig 1). The mean lethal dose equal to the concentration required to reduce survival by 63% on the exponential part of the survival curve (D_0) is 3 μg/ml (1 hour). A concentration of 10 μg/ml reduced the survivors by >95%. The survival of T_1 cells as a function of duration of treatment with 1 μg/ml is shown in figure 2. Cell kill is a continuously exponential function with about 99.99% cells killed after 38 hours of continuous incubation. Control cells treated for 1 hour with supernatant medium containing the drug obtained at each time point showed a survival similar to that obtained with cells treated for 1 hour with freshly prepared drug. This indicates that VP-16-213 is stable in culture medium at 37°C for at least 36 hours. When synchronized cells were treated with VP-16-213 for 1 hour at selected points throughout the length of the cell cycle, cells in S and G_2 phases were considerably more sensitive than

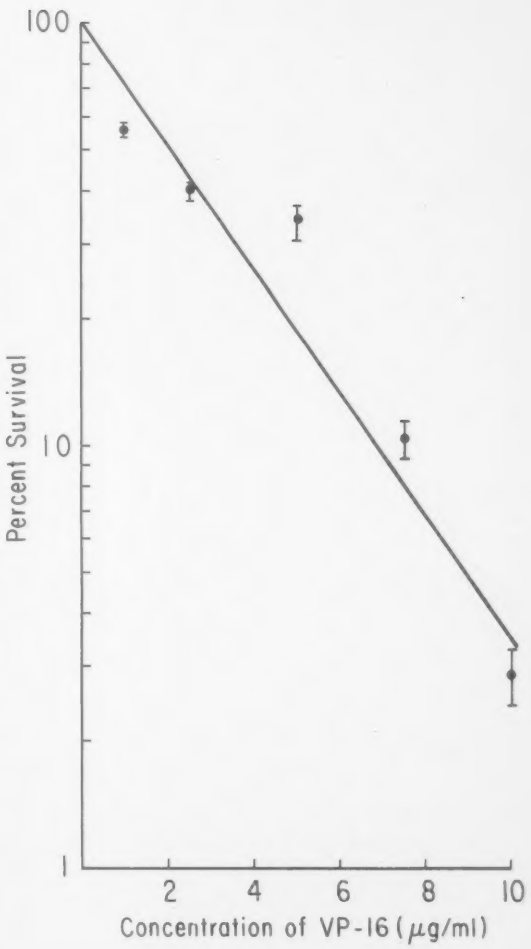

FIGURE 1.—Survival of asynchronous T_1 cells as a function of increasing concentrations of VP-16-213 incubated for 1 hr. Bars represent standard errors.

cells in G_1 phase (fig 3). The shape of the curve was maintained for all drug concentrations.

Cell Cycle Progression

Asynchronous Cells

The effects of concentration and exposure time of VP-16-213 on cell cycle compartment distribution of asynchronous T_1 cells were investigated. An exposure time-dependent increase of the (G_2 + M) fraction was observed for all concentrations of VP-16-213. The MI (0.05%–0.1%) was less than that of control values (0.4%–1.1%) indicating that the com-

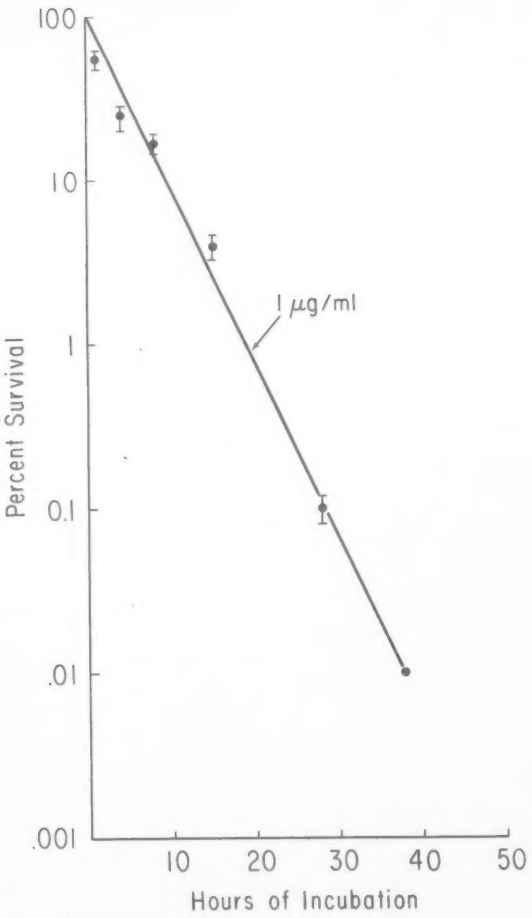

FIGURE 2.—Survival of T_1 cells exposed to 1 μg/ml of VP-16-213 for increasing time intervals. Bars represent standard errors.

partment increase was primarily due to accumulation of cells in G_2 phase.

During continuous incubation with 0.1 μg/ml, a steady increase of cells in G_2 to 92% at 48 hours was observed. Concurrently, both G_1- and S-phase fractions decreased to minimal values of 6% and 2% respectively. Drug removal and incubation with fresh medium at earlier time points (1, 3, and 12 hours of continuous exposure) resulted in considerably smaller compartment changes when compared to the long-term incubation of 48 hours. No significant correlation between duration of treatment for 1, 3, and 12 hours and the subsequent extent of the G_2 block could be seen; in all instances, the population distribution returned to control values about 48 hours after drug release. No discrepancy was found

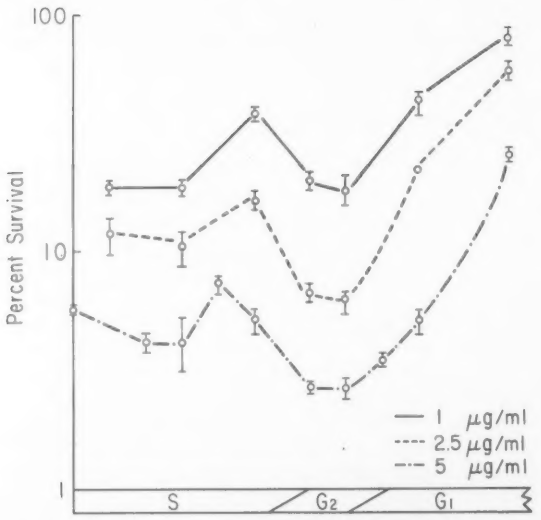

FIGURE 3.—Survival of synchronized T_1 cells exposed for 1 hr to VP-16-213 at selected stages of the cell cycle. Bars represent standard errors.

between the size of the S-phase compartment determined by PCP and the LI for all four exposure times.

Incubation with 0.5 μg/ml (fig 4) induced a steady increase of cells in G_2 during continuous drug exposure for 48 hours, with a maximum accumulation similar to that achieved after treatment with 0.1 μg/ml (95%). Both G_1- and S-phase compartments were almost completely evacuated at 48 hours (ie, G_1 = 3% and S = 4%). The rate of G_2 accumulation and G_1 efflux during the 48-hour exposure was equal to that observed for treatment with 0.1 μg/ml. The S-phase fraction showed a transient slight increase peaking 12 hours after drug addition. Earlier termination of drug treatment (after 1, 3, and 12 hours) and subsequent culture in fresh medium showed progressively smaller degrees of G_2 accumulation and G_1 reduction. Thus, a 12-hour treatment induced a G_2 maximum of 50% compared to 25% following a 3-hour incubation. In addition, these short-term drug exposures resulted in a higher degree of reversibility of the G_2 accumulation resulting in a subsequent rapid increase of the G_1 fraction. There was no significant difference between the percent values of the S-phase compartment determined by PCP and the LI for treatment with 0.5 μg/ml of VP-16-213.

The first kinetic perturbation effect observed during continuous incubation with 1.0 μg/ml for 48 hours was a significant transient increase of cells in the S-phase compartment, reaching a maximum of

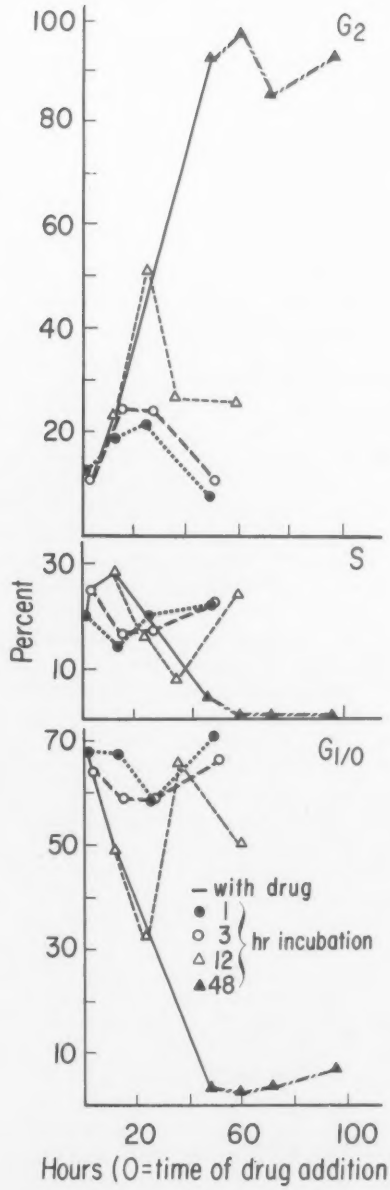

FIGURE 4.—Compartment distribution (PCP) of asynchronous T_1 lymphoma cells treated with 0.5 μg/ml of VP-16-213 for different exposure times. The solid lines indicate the presence of the drug. The dotted lines represent values obtained after drug release. Accumulation of cells in G_2 phase occurs mainly at the expense of cells in $G_{1/0}$ and as a function of duration of treatment.

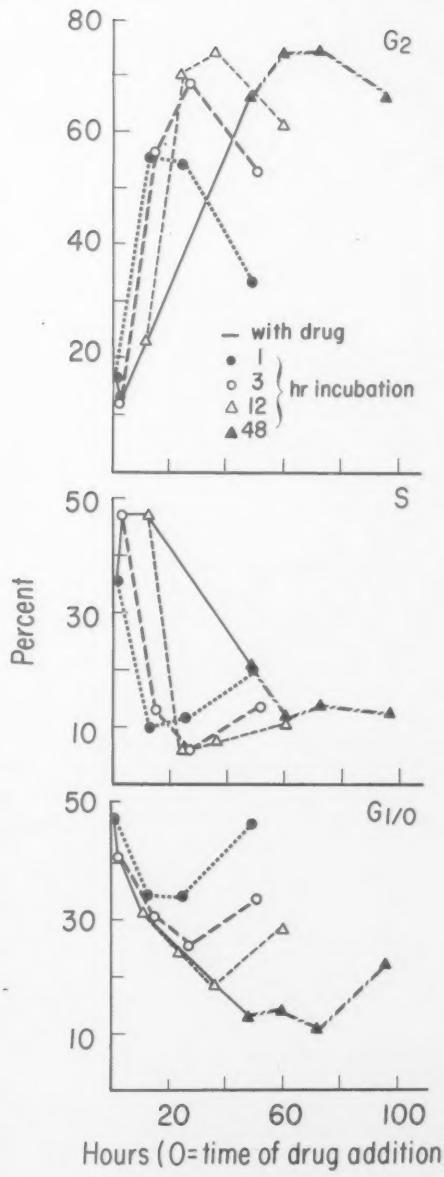

FIGURE 5.—Compartment distribution (PCP) of asynchronous T_1 lymphoma cells treated with 5.0 μg/ml of VP-16-213 for different exposure times. The solid lines indicate the presence of the drug. The dotted lines represent values obtained after drug release. As with 1.0 μg/ml for treatment > 3 hrs, there is a delay in G_2 accumulation in the presence of prompt efflux out of $G_{1/0}$ due to transient delay in S-phase transit.

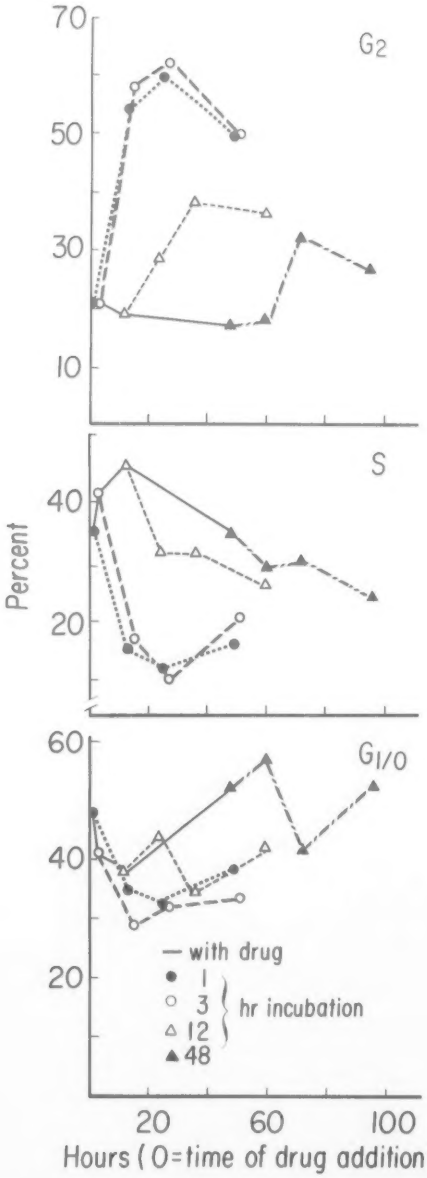

FIGURE 6.—Compartment distribution (PCP) of asynchronous T_1 lymphoma cells treated with 10.0 μg/ml of VP-16-213 for different exposure times. The solid lines indicate the presence of the drug. The dotted lines represent values obtained after drug release. Compared to lower concentrations, the magnitude of compartment shifts is substantially reduced. Only short-term treatment results in an effective G_2 accumulation, mainly at the expense of the S-phase compartment. Prolongation of treatment for 12 and 48 hrs induces a comparatively small accumulation of cells in G_2 phase, following a transient increase of the S-phase compartment.

60% after 12 hours. Concurrently, the G_1 compartment decreased, but no change of the G_2 fraction was noted. The final histogram distribution at the end of the 48-hour treatment was not different from the distribution pattern obtained after treatment with 0.1 and 0.5 μg/ml. Drug release after either the 1-, 3-, or 12-hour treatment resulted in an immediate evacuation of the S-phase compartment followed by a prompt increase of the G_2 fraction. Peaks of G_2 accumulation were distinctly correlated to the duration of treatment (1 hour = 42%; 3 hours = 57%; and 12 hours = 65%). Similarly, the extent of reversibility of G_2 accumulation (with subsequent rise in G_1) was also related to the time of drug exposure.

Further increase of drug concentration to 5 μg/ml (fig 5) showed profiles for G_1, S, and G_2 curves similar to those observed for treatment with 1.0 μg/ml. Yet, in contrast, the slope and magnitude of G_2 accumulation during continuous 48-hour treatment were reduced, and a G_2 maximum of 75% was achieved 12 hours after drug release. Concomitantly, there were somewhat larger residual populations in both G_1 and S phase (about 10% each). Drug removal after 1, 3, and 12 hours did not accelerate the evacuation of the G_1 compartment when compared to prolonged treatment. G_2 peak values attained after these short-term drug exposures were again closely correlated to duration of treatment.

The highest drug concentration studied was 10 μg/ml. Continuous drug exposure for 48 hours induced only a slight and transient increase in the size of the S-phase compartment at the expense of the G_1 population. There was no increment of G_2 cells (fig 6). Twenty-four hours after drug release, ie, at 72 hours after drug addition, a small decrease of the G_1 compartment and a similar increase of the G_2 fraction were observed. Drug release after 1- and 3-hour treatments induced an immediate increase of the G_2 compartment (60%) which was only slightly smaller compared to that obtained with the lower concentrations of VP-16-213. At the end of the 12-hour incubation, the increase of cells in S phase was primarily due to accumulation in early S phase (fig 7*a*). During the subsequent 12-hour culture period in drug-free medium, a major shift from early into late S phase (and further into G_2 phase) had occurred (fig 7*b*). Twelve hours later the G_2 maximum of 38% was reached (fig 7*c*). Thus, when compared to treatment with lower VP-16-213 concentrations, an opposite pattern of the effect of duration of treatment on maximal G_2 accumulation was observed.

LI values were significantly smaller than the corresponding S-phase fractions determined by PCP when treatment with 10 μg/ml was extended for > 3 hours. Whereas the discrepancy between PCP-determined S fraction and LI values (S/LI = 2) at the end

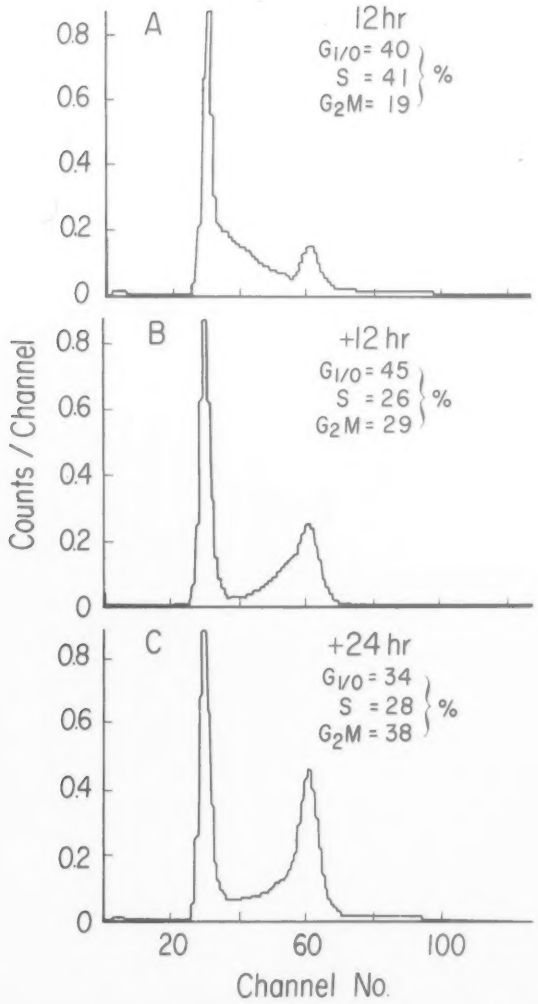

FIGURE 7.—DNA histograms (PCP) of asynchronous T_1 lymphoma cells treated with 10 μg/ml of VP-16-213 for 12 hrs. A = the end of 12-hr drug exposure; B = 12 hrs after drug release; and C = 24 hrs after drug release. Notice the accumulation of T_1 cells in early S phase (*A*), a subsequent shift of this cohort into late S and G_2 phase (*B*), and the final accumulation in G_2 phase (*C*).

of a 3-hour incubation was resolved during the subsequent 12-hour incubation in drug-free medium, the difference at the end of a 12- and 48-hour treatment was maintained over the ensuing 48 hours after drug removal (fig 8).

Figure 9 summarizes the data on the peak values of G_2 accumulation obtained, regardless of whether this peak was observed during drug exposure or in the subsequent 48 hours following drug removal. The time of appearance of the peak was neglected in

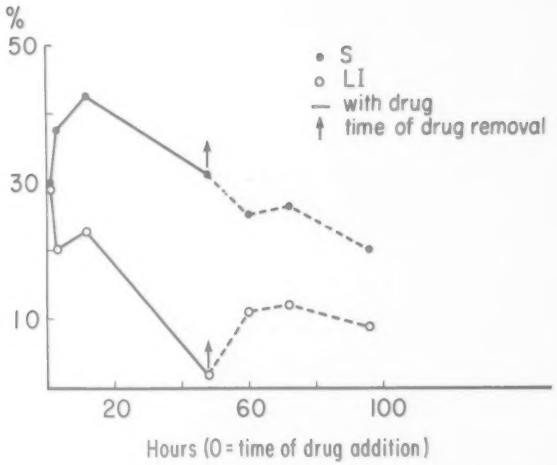

FIGURE 8.—Asynchronous T_1 lymphoma cells were treated with 10 μg/ml of VP-16-213 for 48 hrs. After drug release, 2 washings with Hanks' balanced salt solution, and replacement of fresh medium, cells were harvested 3 more times up to 96 hrs from drug addition. There is a marked dissociation of S-phase fraction (PCP) and LI (^{3}H-TdR), with temporary complete block of DNA synthesis at 48 hrs and partial resumption after drug release.

order to aid the visualization of the blocking effect of VP-16-213. For concentrations ≤ 1.0 μg/ml, prolongation of drug exposure results in a continuous increase of the extent of G_2 accumulation, with peak values observed after 48 hours of uninterrupted incubation. Perturbation with 5 μg/ml leads to a plateau of 70% after a 3-hour incubation. Prolongation of treatment with 10 μg/ml beyond 3 hours adversely affects the kinetic response with a decrease of the G_2 maximum to 40% after a 12-hour incubation and 33% after a 48-hour drug exposure.

To test further fluctuations in the compartment distribution subsequent to recovery from G_2 accumulation, long-term followup experiments over a total period of 200 hours were carried out. After perturbation with 1 and 5 μg/ml for 3 hours, second waves for S (at 48 hours) and for G_2 (at 72 hours) compartments were observed (fig 10).

Synchronized Cells

Synchronized cells were exposed to 5 and 10 μg/ml of VP-16-213 for 1 hour throughout the cell cycle. Compartment distribution was monitored for 180 hours after release of TdR block. When DNA histograms were measured at the time the control population had reached the G_2 phase, cells treated while in early, mid, and late S phase with 5 μg/ml showed the same compartment distribution as controls. Treatment with 10 μg/ml, however, resulted in a decreased G_2 compartment for cells which had been treated in mid S phase; 70% of the cells were in late

S phase and only 20% in G_2 (fig 11). Further followup at 30 hours after TdR release showed 85%–90% of the population in G_2 phase, independent of the time of initiation of treatment in the various stages of DNA synthesis.

Cells treated in all phases of the cell cycle with 5 and 10 μg/ml of VP-16-213 were effectively accumulated in G_2 phase (figs 12 and 13). The maximal values of G_2 accumulation during observation periods ≤ 180 hours after TdR release are indicated by the height of the bars. The degree of reversibility of this kinetic effect is shown by the dotted segments. A marked difference between the two concentrations becomes apparent; the extent of recovery from G_2 accumulation decreases with increasing concentrations for cells treated in G_2. Thus, independent of initiation of treatment in either mid and late S phase or in early and late G_2 phase, similar residual G_2 populations (slashed segments) were observed after treatment with 10.0 μg/ml. However, the residual G_2 population of cells treated while in early S phase was smaller when incubated with 10 μg/ml (35%) compared after exposure to 5 μg/ml (60%). G_1 cells were least affected by both drug concentrations. The time interval for recovery from the G_2 block varied. At 5 μg/ml cells treated in G_2 showed the fastest reversibility (10–15 hours) followed by

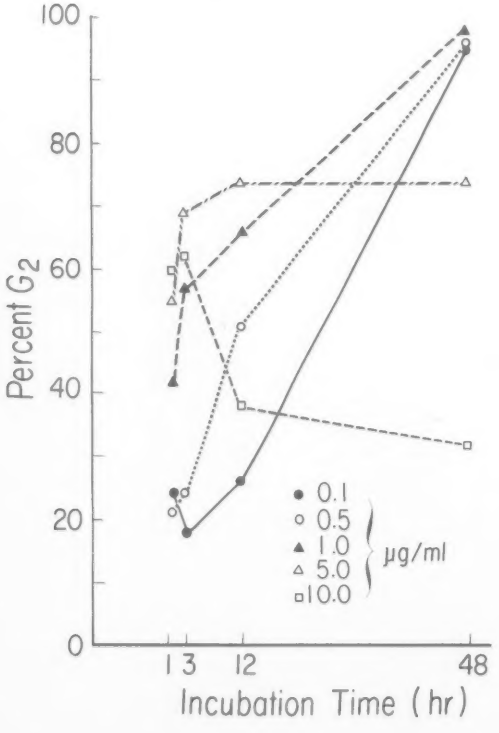

FIGURE 9.—Maximum G_2 accumulation of asynchronous T_1 lymphoma cells, regardless of time of appearance, as a function of duration of treatment with VP-16-213 for different concentrations. Note that concentrations > 5 μg/ml and prolongation of treatment for > 3 hrs reduce the magnitude of G_2 accumulation. The percent of cells in G_2 phase of control cultures varied from 10% to 18% throughout the experiment.

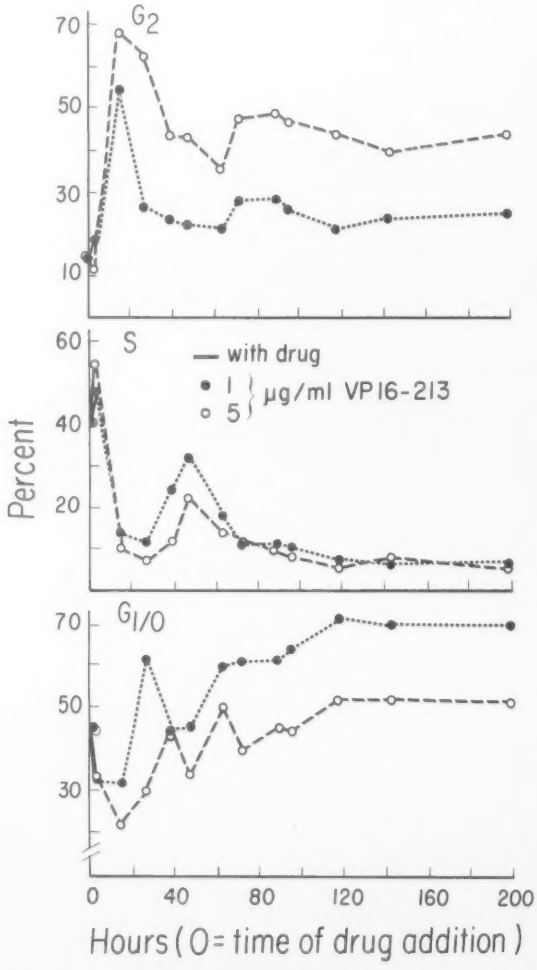

FIGURE 10.—Compartment distribution (PCP) of asynchronous T_1 lymphoma cells treated with 1 and 5 μg/ml of VP-16-213 for 3 hrs. Following a transient increase in the S-phase fraction, there was a concentration-dependent G_2 accumulation with subsequent increase of cells in $G_{1/0}$, S, and (G_2 + M) fractions.

populations treated in late, mid, and early S phase. After treatment with 10 μg/ml in early S phase, 30% of the accumulated population had recovered at 30 hours after TdR release; the other populations reached the minimal G_2 values around 100 hours after TdR release.

DISCUSSION

Podophyllotoxin derivatives have been classically considered mitotic poisons (3,8) that produce an accumulation of cells in mitosis. However, the new semisynthetic podophyllotoxin derivatives VM-26 and VP-16-213 differ in their biologic properties in that they produce a decrease of the MI suggesting that they act primarily at the premitotic stage (2,6,9,10,15). Although cell proliferation decreases after incubation with low concentrations of VP-16-213, effects on macromolecular synthesis are only noted at higher concentrations (1) suggesting that the lethal mechanism of action is not related to inhibition of either nucleic acid or protein synthesis. Studies on the effects of VP-16-213 on inhibition of cellular proliferation and macromolecular synthesis and on the induction of chromosomal aberrations have indicated that the main effect of VP-16-213 is produced in late S and G_2 phases (1,15).

We investigated the effect of VP-16-213 on the reproductive capacity of human lymphoma cells using concentrations similar to those employed by the above-mentioned investigators. The lethal effect of VP-16-213 (D_o = 3 μg/ml, 1 hour) is one of the most powerful manifested thus far by numerous chemotherapeutic agents studied on this cell line. Only adriamycin (D_o = 0.15 μg/ml, 1 hour) has a greater lethal effect on T_1 cells (16). VP-16-213 appears very stable in growth medium and induces a continuous logarithmic kill as a function of incubation time. A concentration of 1 μg/ml reduces the survivors by 1000-fold after a period equal to the length of one cell cycle. Although the shape of our survival curve was different from the biphasic shape noted by Stähelin (17) for mouse leukemia cell lines, in a fashion similar to that of his results, the drug concentration played a more important role in re-

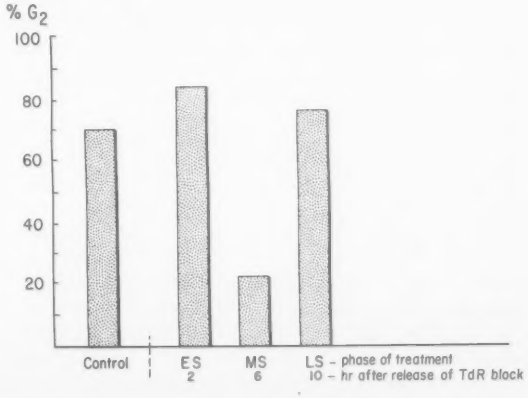

FIGURE 11.—TdR-synchronized T_1 lymphoma cells were treated with 10 μg/ml of VP-16-213 for 1 hr in early S (ES), mid S (MS), and late S (LS). Compartment distribution (PCP) was determined at the time of EG_2 for control populations (13 hrs). The G_2 compartment size of cells treated while in ES and LS was equal to that of controls. When treatment was initiated in MS, S-phase transit was delayed so that only 20% of the cells had progressed into G_2 at 13 hrs.

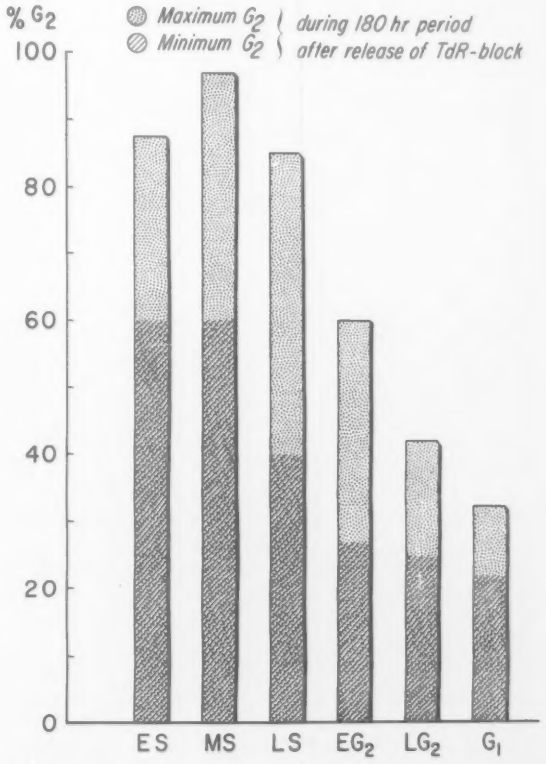

FIGURE 12.—Maximum G_2 accumulation (bar height) of T_1 lymphoma cells after treatment with 5 μg/ml of VP-16-213 for 1 hr as a function of cell age. When treatment was initiated while cells were in S phase, an accumulation of 85%–95% of cells in G_2 phase was obtained. Cells treated while in G_2 and G_1 phase were less effectively blocked in G_2 in this sequence. The dotted segments of the bars indicate the percent recovery from G_2 accumulation during the 180-hr period after TdR release. The residual irreversibly blocked G_2 fraction is largest when treatment was initiated in S phase. Control cultures had a maximum of 78% cells in G_2 at 13 hrs and a minimum value of 16% at 28 hrs (time of mid G_1 phase).

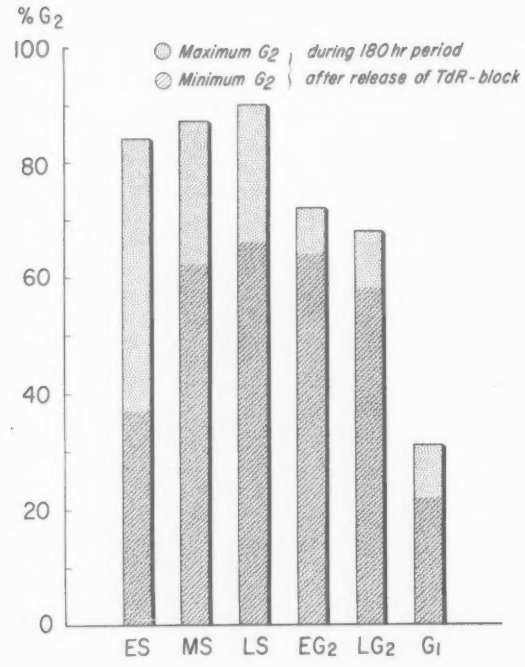

FIGURE 13.—Maximum G_2 accumulation (bar height) of T_1 lymphoma cells after treatment with 10 μg/ml of VP-16-213 for 1 hr as a function of cell age. Compared to 5 μg/ml (fig 12), cells treated while in G_2 phase were more effectively blocked in G_2 with only little recovery resulting in similar residual G_2 fractions for populations treated in MS, LS, EG_2, and LG_2. G_1 cells were least effectively blocked. Notice the high degree of reversibility for cells treated in ES.

ducing cell survival than did the interval of incubation. Thus, cell survival after a 10-hour incubation with 1 μg/ml was threefold less than that obtained with 10 μg/ml for a 1-hour treatment.

T_1 cells manifested cell cycle stage-dependent sensitivity to VP-16-213. Although the drug killed cells in all phases it was more efficient in late S and G_2. Almost a tenfold difference in killing efficiency was noted from the lowest survival point in mid G_2 to the highest survival point in mid G_1. Thus, cell-killing data support the cell cycle dependency of VP-16-213 effects noted for other biologic events.

A moderate discrepancy was noted between the proportion of cells in the various compartments defined by PCP and the expected percentages derived from the values of cell cycle stage transit times defined by the PLM method. It should be remembered that these two methods measure different parameters, and therefore moderate discrepancies can be expected. Mean values obtained by the PLM technique are biased by the transit times of rapidly proliferating cells and do not take into account the proportion of cells within the population that may have a long G_1 or actually be in a G_0-like state. In a previous publication (18), we reported that the generation time of T_1 cells may range from < 20 to > 60 hours, and that a significant fraction of cells (10%–20%) do not cycle during the average cell cycle time. Furthermore, we also indicated that the length of G_2 phase of T_1 cells may actually be as short as 6 hours, the longer duration defined by the PLM method being the result of a technical artifact induced by the washing procedures following the pulse labeling. Those results have been recently confirmed by extensive studies utilizing time-lapse photography and will be reported in a subsequent manuscript. The length of S phase and the generation time are free of this artifact, and thus the length of G_1 is correspondingly increased by 4 hours. If all of these facts are taken into account (ie, that T_1 cells have an average generation time of 31 hours, that the true cell cycle stage times are 15 hours for G_1, 10 hours for S, and 6 hours for G_2, and that under unperturbed conditions about 80% of the cells cycle during the average generation time), the actual expected proportion of cells in the various compartments are: G_2, 15.5%; S, 25.8%; and G_1, 38.7%. Since PCP measures both G_1 and G_0 cells as pertaining to one compartment, the value of 58.7% is to be expected for this compartment. Thus, the compartment distribution size measured by PCP for T_1 cells corresponds fairly closely with the expected values derived in reference to cell cycle stage times defined by the PLM technique. This is further supported by the fact that the LI of asynchronously growing T_1 cells is 26 (range, 22–29). Perturbation with drug treatment (including TdR used for synchronization) changes the proportion of cells with long G_1 phases or cells in G_0. Hence, almost 90% of cells in S phase can be obtained by our routine synchronization procedures and occasionally almost complete evacuation of the $G_{1/0}$ compartment can be seen following treatment with certain drugs.

In our studies, VP-16-213 induced a concentration- and exposure time-dependent, highly reversible delay of the cell cycle progression in G_2 phase. For concentrations ≤ 1.0 μg/ml, prolongation of drug treatment results in progressive increments of the magnitude and duration of the G_2 accumulation. Similarly, there is a positive correlation with the size of the irreversibly arrested G_2 population. Perturbation with 5 μg/ml elicits a plateau-type curve of G_2 maximum accumulation; compared to lower concentrations, there is an effective G_2 delay after short-term incubation (1–12 hours) with no further increase following extension of treatment for 48 hours. At a concentration of 10 μg/ml, treatment for

> 3 hours adversely affects both the onset and the magnitude of G_2 accumulation.

As can be inferred from the equal initial slopes of G_1 compartment evacuation both during long-term continuous incubation (48 hours) and during and after short-term exposure (1–12 hours), neither the concentration nor the duration of treatment for dose levels < 5 μg/ml alter the rate of efflux out of G_1. Treatment with 5 μg/ml of VP-16-213 decreases the rate of exit out of G_1, leaving a residual compartment of 10% in G_1. Perturbation with 10 μg/ml allows only a small population of 15% to traverse from G_1 into S, only after short drug exposure (1 and 3 hours), whereas longer incubation (12 and 48 hours) blocks 40%–50% of the population in G_1. The transient increase of the S-phase population, most pronounced after treatment with 5 μg/ml, indicates a delay in S-phase transit. Yet, the S-phase compartment is completely evacuated during the 48-hour treatment interval with 1 μg/ml. Earlier drug removal results in immediate suspension of the S-phase block, allowing more rapid accumulation in G_2 phase. The absence of discrepancy between the percent S-phase fraction (PCP) and the LI for concentrations < 10 μg/ml indicates that the progression through S phase is only slowed down but not completely blocked. When exposed to 10 μg/ml for > 3 hours, DNA synthesis is markedly reduced. The increase of cells in S phase from 30% to 45% during continuous incubation with 10 μg/ml indicates influx out of G_1. Thus, initiation of DNA synthesis is not immediately suppressed. The resulting discrepancy between LI- and S-phase fraction (eg, at the end of 48-hour incubation, PCP = 30% and LI = 1%) along with only minor compartment changes after incubation for 12 and 48 hours reflects a considerable immobilization of cells throughout the cycle.

Cell age where treatment was applied proved to be a determinant of the subsequent kinetic response; although transit prolongation through the immediate S-phase compartment after treatment of cells in mid S phase with 10 μg/ml was noted, G_2 phase was the only site of significant progression delay induced by a 1-hour perturbation with 5 and 10 μg/ml. Under these conditions, cells treated in S phase were most effectively arrested in G_2 (80%–90%). The magnitude of G_2 accumulation for cells treated in G_2 phase was considerably less (40%–70%). Perturbation of cells in G_1 phase induced only a minor transient G_2 accumulation of 30%.

The high degree of recovery from G_2 block with subsequent cell division does not imply unimpaired cell reproduction, as assessed by colony formation. VP-16-213, like bleomycin (14), affects cell cycle traverse of the immediate life cycle only transiently and partially. However, the progeny retains only limited reproductive potential. Cell survival (colony formation) did not correlate with amplitude or duration of the G_2 block at any concentration of VP-16-213; yet, the stage of maximum lethality was also G_2 phase.

VP-16-213 caused a delay in G_2 transit similar to that induced by several other drugs studied on this cell line (19). This block of G_2 traverse may be the result of a direct effect on one or several hypothetical "division-specific" enzymes (factor Y) or on one of a series of precursor molecules (factor X). The synthesis of both factors may take place throughout the cell cycle, but it is the level of factor Y at the time of G_2 phase which determines the traverse speed through that stage. Our data suggest that VP-16-213 affects both factors X and Y, although this effect is relatively short-lived. Treatment in G_1 phase resulted in a negligible G_2 block, while incubation in both S and G_2 phases resulted in sustained delay. Thus, VP-16-213 behaves differently than Yoshi 864, another chemotherapeutic agent studied on this cell line (20). Although Yoshi 864 induced a G_2 block after treatment in G_1 and early S, it failed to delay transit through G_2 if applied in late S and G_2 phases. These types of differential effects caused by antitumor agents could, perhaps, be used to identify the nature of the postulated division-related factors.

It is noteworthy that VP-16-213, in addition to its effect on G_2 transit, exerts inhibitory effects at other stages of the cell cycle. With concentrations of 10 μg/ml administered for > 3 hours, a "frozen state" of the whole life cycle was observed. Thus, with increasing concentrations and exposure times, the capacity of VP-16-213 to interfere with different target molecules increases.

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Studies With 2,5-Piperazinedione, 3,6-Bis(5-chloro-2-piperidyl)-, Dihydrochloride. I. Cell Kinetic and Biologic Effects in Cultured L1210, Human Epidermoid No. 2, and Adenocarcinoma 755 Cells[1,2,3]

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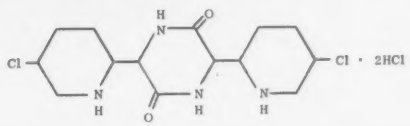
SUMMARY

Experimental results obtained with cultured L1210, human epidermoid No. 2, and Adenocarcinoma 755 cells are consistent in showing that inhibition of proliferation of the cells by 2,5-piperazinedione, 3,6-bis(5-chloro-2-piperidyl)-, dihydrochloride is accompanied by cell enlargement. Although cells initially in the G_2 phase when exposure to the agent is begun can probably proceed through mitosis and divide, cells that are initially in G_1 and S phases accumulate in the G_2 phase. Progression of cells to G_2 phase during the period of exposure to the agent is not a requisite for cell-killing, because cells exposed for periods insufficient to permit their accumulation in G_2 are killed.

[Cancer Treat Rep 60:1307–1316, 1976]

2,5-Piperazinedione, 3,6-bis(5-chloro-2-piperidyl)-, dihydrochloride (NSC-135758) was isolated from fermentation broths of *Streptomyces griseoluteus* by Gitterman and his associates (1). Arison and Beck (2) established its structure:



This agent retarded the growth of a human tumor in the human tumor-egg host system (1) and it retarded the growth of several experimental neoplasms (3). It also elicited favorable responses in several neoplasms in the clinic (4). Because of these results, we have performed a variety of experiments to learn more about the biologic, biochemical, and chemotherapeutic effects of this agent. The effects of NSC-135758 upon the rate of proliferation, size, progression through the cell cycle, and viability of cultured mammalian cells are reported in this paper. The biochemical and chemotherapeutic effects are reported in the two following reports in this issue.

MATERIALS AND METHODS

Cell Culture

Three kinds of cultured mammalian cells were used in these studies: murine L1210 leukemia, human epidermoid carcinoma No. 2 (H.Ep. No. 2), and murine Adenocarcinoma 755.

Since some of the experiments with L1210 cells were performed at the NCI and some at the Southern Research Institute, two cell lines grown under different conditions were used. The cells used for flow microfluorometry were grown in RPMI-1630 medium supplemented with 20% fetal calf serum, penicillin, and streptomycin. The cells used to determine the effects on proliferation, size, and viability were derived from ascites cells taken from a mouse

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[2]Supported by contract N01-CM-43784 from the Division of Cancer Treatment (DCT), National Cancer Institute (NCI), National Institutes of Health, Department of Health, Education, and Welfare.

[3]2,5-Piperazinedione, 3,6-bis(5-chloro-2-piperidyl)-, dihydrochloride: NSC-135758; CAS reg. No. 41109-80-2. Supplied by the Drug Research and Development Program (DR&DP), DCT, NCI.

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and cultured initially in stationary sealed flasks of Dulbecco's medium containing mercaptoethanol (5 μM) and supplemented with 10% horse serum. After being serially cultured in this medium for some time they were subsequently grown in Fischer's medium supplemented with 10% horse serum in sealed stationary Erlenmeyer flasks. When these latter cells were inoculated ip into mice, the time to death of the recipient mice was essentially the same as that for cells transferred directly from mouse to mouse.

The H.Ep. No. 2 cells were maintained in stock as swirl cultures in SRI-14 medium (5) supplemented with 10% calf serum. For exposure of the cells to the test agent, approximately 100,000 cells and 5 ml of medium containing serum and 25 μg/ml of calcium chloride were placed in 1-oz prescription bottles which were closed and laid on their flat sides in an incubator. The medium was decanted from the glass-attached cells and replaced with fresh medium at 24 and 48 hours, and the agent was added with the medium at 48 hours.

Adenocarcinoma 755 cells were grown overnight in SRI-14 medium supplemented with 10% calf serum in 30-ml swirl cultures. The test compound was added to the cultures after this initial incubation period, and at this time the population density was approximately 10^5 cells/ml. Incubation was then continued for specified time periods.

Enumeration of Cells

The study utilizing L1210 cells and the Cytofluorograf were performed at the NCI. In experiments making up that study, both living and formaldehyde-fixed cells were diluted in phosphate-buffered saline and counted with a Coulter Electronic Particle Counter with an orifice diameter of 100μ, 1/amplification of 4, 1/aperture current of 0.5, a lower threshold at 10 scale units, and an upper limit of infinity.

In experiments with L1210 cells performed at the Southern Research Institute, 1.0 ml of the cell suspension was added to 18.5 ml of SRI counting solution, and 0.5 ml of formaldehyde was added to this suspension. (SRI counting solution consists of SRI-14 salts solution [5] containing 0.05% penicillin, 0.05% streptomycin, and 0.125% trypsin 1:300.) The cell count was then determined with a Model B Coulter Electronic Particle Counter with a tube having a 100μ diameter orifice and with a 1/amplification setting of 2, a 1/aperture current setting of 0.707, a lower threshold at 20 scale units, and an upper limit of infinity.

The H.Ep. No. 2 cells were harvested from the prescription bottles by decanting and retaining the supernatant medium, incubating the cell layer with 3 ml of SRI counting solution containing 0.05% trypsin 1:300 (instead of 0.125%) at 37°C for 10 minutes, shaking to disperse the cells, adding this suspension plus two 2-ml 0.9% NaCl rinsings of the bottle to the retained supernatant medium, and centrifuging in a clinical centrifuge. The sedimented cells were washed two times with 5 ml of SRI salts solution and resuspended in 10 ml of SRI-14 medium containing $CaCl_2$. Two ml of this suspension was added to 17.5 ml of counting solution, and 0.5 ml of formaldehyde was added. The cell count was determined with the Coulter Counter with a 1/amplification setting of 4, a 1/aperture current setting of 2, a lower threshold setting of 25 scale units, and an upper limit of infinity.

One ml of the Adenocarcinoma 755 culture was added to 18.5 ml of SRI counting solution containing 0.05% 1:300 trypsin, and the mixture was incubated at 37°C for 1 hour. After the addition of 1 ml of formaldehyde, the cells were counted with the Coulter Counter with a 1/amplification setting of 4, a 1/aperture current setting of 1, a lower threshold of 25 scale units, and an upper limit of infinity.

Determination of Size Distribution

The samples of the three kinds of cells were prepared as described above for cell enumeration. The Coulter Counter was used to determine the size distribution of the cells using counting windows equal to 5 scale units and using the following settings of 1/amplification and 1/aperture current respectively for the different kinds of cells: L1210, 1 and 8; H.Ep. No. 2, 32 and 1; Adenocarcinoma 755, 16 and 0.707.

Determination of Viability

The viability of the L1210 cells was determined by bioassay in mice (6). Samples of untreated and treated cultures of cells were diluted with medium to a cell density of 10^5 cells/ml, and 1 ml was injected ip into each of five mice. The survival times of the recipient mice were determined and compared with the survival times of mice that had received serial logarithmic dilutions of untreated cultured cells.

The viability of H.Ep. No. 2 cells was determined by the cloning method. Portions of the suspensions of harvested and washed cells in SRI-14 medium containing $CaCl_2$ that were used above for the enumeration of cells were diluted to contain 1000 or 100 cells/10 ml, and 10 ml of these suspensions was placed in 25-cm^2 plastic culture bottles in triplicate. After incubation at 37°C for 12 days, the medium was decanted, the cells were rinsed with phosphate-buffered saline, fixed with Bouin's solution, and

stained with Giemsa, and macrocolonies were counted.

The viability of the adenocarcinoma cells was not determined.

Determination of Effects Upon Progression Through the Cell Cycle

L1210 cells were grown in RPMI-1630 medium supplemented with 20% fetal calf serum, penicillin, and streptomycin. After 6-, 12-, and 24-hour periods of drug exposure, aliquots of untreated and drug-treated cultures were fixed and stained with benzoflavine for flow microfluorometry using a procedure modified from that employed by Tobey et al (7). Modifications consisted of: (*a*) elimination of the trypsin/deoxyribonuclease digestion prior to fixation; (*b*) addition of 0.5% Triton-X-100 to all solutions utilized in the staining procedures to enhance monodispersion; (*c*) use of 0.02% benzoflavine in lieu of 0.03% acriflavine; and (*d*) use of an equal number of cells for each sample during the staining process.

The frequency distribution of fluorescence emission per cell (proportional to DNA content) was measured for samples of 10^5 cells using a Cytofluorograph (Biophysics Systems, Inc, Baldwin Place, NY) and analyzed with a multichannel analyzer (Nuclear Data Inc, Madison, Wis). The results are expressed as a histogram representing the relative number of cells with a given DNA content.

The colcemid-block method for continuous exposure of the cells to the agent and to C^3H_3-deoxythymidine (8) was used to determine the effects of the agent upon the progression of H.Ep. No. 2 cells through the cycle. This method is based upon measurement of the rate of cells becoming labeled and the rates of accumulation of unlabeled and labeled cells at mitosis.

RESULTS

Effects on L1210 Cells

Figure 1 shows the extent of proliferation of L1210 cells during 48 hours in the presence of four concentrations of NSC-135758. The control culture grew exponentially for about 24 hours with a doubling time of approximately 14 hours, and then the growth rate decreased. In the presence of the drug at all four concentrations, some of the cells divided, but the number of cells in the treated cultures did not double during the 48-hour period of the experiment. Figure 2 shows that enlargement of cells occurred even during the first 6 hours of exposure to all concentrations of the agent and that greater enlargement occurred with the higher concentrations. By 24 hours, some of the cells exposed to the lower concentrations were even larger, but the presence of large numbers of small particles is consistent with the possible occurrence of cell fragmentation. The high percentage of small particles and the absence of a maximum in the curve for 5 μg/ml after an exposure of 24 hours are suggestive of extensive fragmentation of cells. The bioassay data of table 1 show that < 1% of the cells that were exposed to the drug at a concentration of 0.1 μg/ml for 6 hours remained viable upon implantation in mice, and none of the cells exposed to this concentration for 24 hours or cells exposed to the higher concentration for either 6 or 24 hours retained viability.

The effects of NSC-135758 on the progression of L1210 cells through the cycle were evaluated by

TABLE 1.—Bioassay of cultured L1210 cells after exposure to NSC-135758

No. of cells implanted	Concentration (μg/ml)	Time of exposure (hr)	Day of death		40-Day survivors	Approximate No. of surviving cells
			Mean	Median		
8.5×10^5	—	—	8.2	8	0/5	8.5×10^5
1×10^5	—	—	9.2	9	0/5	1×10^5
1×10^4	—	—	10.2	10	0/5	1×10^4
1×10^3	—	—	11.8	12	0/5	1×10^3
1×10^2	—	—	12.6	13	0/5	1×10^2
1×10^5	0	6	10.4	10	0/5	1×10^4
1×10^5	0.1	6	17.8	16	0/5	$< 1 \times 10^3$
1×10^5	0.3	6	—	—	5/5	0
1×10^5	1.0	6	—	—	5/5	0
1×10^5	5.0	6	—	—	5/5	0
1×10^5	0	24	11.4	11	0/5	1×10^3
1×10^5	0.1	24	—	—	5/5	0
1×10^5	0.3	24	—	—	5/5	0
1×10^5	1.0	24	—	—	5/5	0
1×10^5	5.0	24	—	—	5/5	0

flow microfluorometry; results are shown in figure 3. Exponentially growing L1210 cells show the patterns seen in part A of figure 3. The relative DNA content has been scaled to a value of 1.0 for the first peak which is produced by cells with a G_1-phase DNA content. The second peak represents cells with a G_2-phase DNA content and the cells between these two peaks have the increasing DNA contents of S-phase cells. The tail of cells with DNA values > 2 is an artifact produced by doublet cells. Cells continuously exposed to either 0.4 μM (0.17 μg/ml; fig 3, B) or 3.0 μM (1.27 μg/ml; fig 3, C) grew respectively at 55% and 35% of the control rate. After 6 hours, only small changes in the distribution of cells were seen, primarily a decrease in the number of G_1-phase cells seen at the higher concentration. By 12 hours the G_1-phase population had moved into S phase. At this time, a shoulder of G_1 cells was seen at the lower concentration because of the presence of a larger population of G_1 cells than at the higher concentration. This is due to a higher rate of cell division at the lower concentration, producing G_1 cells at a greater rate. By 24 hours, the major effect of the drug was seen as an accumulation of cells with a G_2-phase DNA content. This effect is clearer at the higher concentration. The mitotic index of these cells at 24 hours was not elevated, being 6% and 2% respectively. These results indicate that NSC-135758 arrests cells in G_2 phase rather than in mitosis and that G_1- and S-phase cells are capable of progressing through the cycle to the G_2 phase where they are blocked.

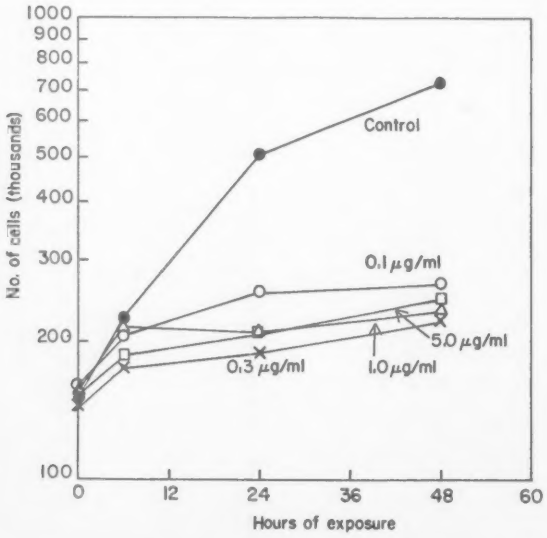

FIGURE 1.—Effects of NSC-135758 upon the proliferation of cultured L1210 cells.

Effects on H.Ep. No. 2 Cells

Figure 4 shows the extent of proliferation of H.Ep. No. 2 cells in the absence and in the presence of NSC-135758. Some proliferation occurred in each of the treated cultures, but in none of the treated cultures did the cell count double during the 48-hour period of the experiment. After exposure to the agent for 6 hours there is evidence of a small increase in the size of some cells (fig 5), and there is somewhat more enlargement by 24 hours. As in the case of the L1210 cells described above, there was some evidence of fragmentation of the cells exposed to the greater concentrations of agent by 48 hours, but there was also an increase in the percent of small particles in the control culture.

The graphs in figure 6 show the effects of the agent upon the progression of H.Ep. No. 2 cells through the cell cycle when initially exponentially growing non-synchronized cells are continuously exposed to it. In order to interpret the graphs it is necessary to know the following experimentally determined lengths of the cell cycle and its phases for these cells under these experimental conditions (8): T_c, 26.1 hours; T_{G_1}, 14.4 hours; T_S, 6.9 hours; T_{G_2}, 3.9 hours; and T_M, 0.9 hours.

Figure 6A shows the rates of accumulation of cells (both labeled and unlabeled) at mitosis. The presence of NSC-135758 at the indicated concentrations had little effect upon the accumulation of cells at mitosis for the first 6–8 hours. This shows that the cells initially in G_2 phase and the latter half of the S phase proceeded to mitosis at the normal rate. After 6–8 hours the arrival of the cells at mitosis was slowed, and the degree of slowing increased with increasing concentration of the agent. When the concentration of the agent was 5.0 μg/ml, very few cells arrived at mitosis between 8 and 32 hours.

Figure 6B shows the rates of accumulation of labeled mitotic cells, and the data are consistent with the data of figure 6A. Theoretically, no labeled cells should reach mitosis until a period of time equal to G_2 (3.9 hours) has passed, and then labeled mitotic cells of the control culture should accumulate at an exponential rate. In this experiment, the values for the control culture at 6 and 8 hours are lower than would be expected, but the results for the control and treated cultures are similar for the first 6–8 hours. After 6–8 hours, the rate of accumulation of labeled mitotic cells decreases with increasing concentrations of the agent.

Figure 6C shows the rate of labeling of cells with C^3H_3-deoxythymidine. Only for the highest concentration (5 μg/ml) of agent is the curve for the treated culture consistently lower than that of the control culture, and even that curve is not much lower. This

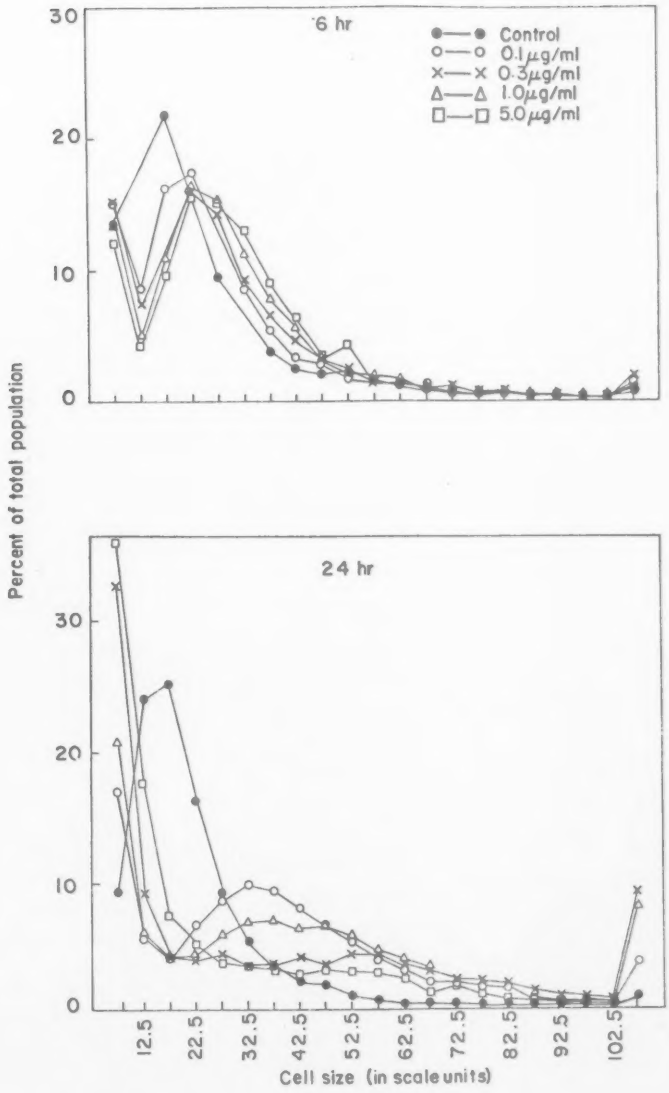

FIGURE 2.—Effects of NSC-135758 upon the size distribution of cultured L1210 cells.

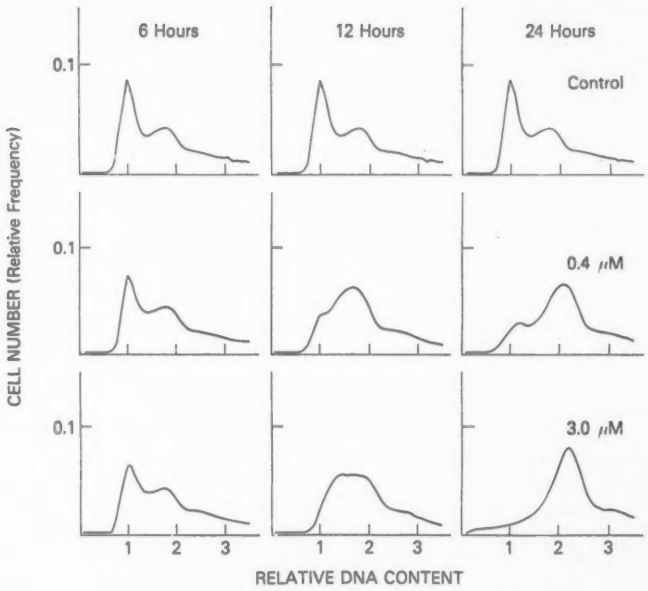

FIGURE 3.—Effects of NSC-135758 upon the DNA content of cultured L1210 cells.

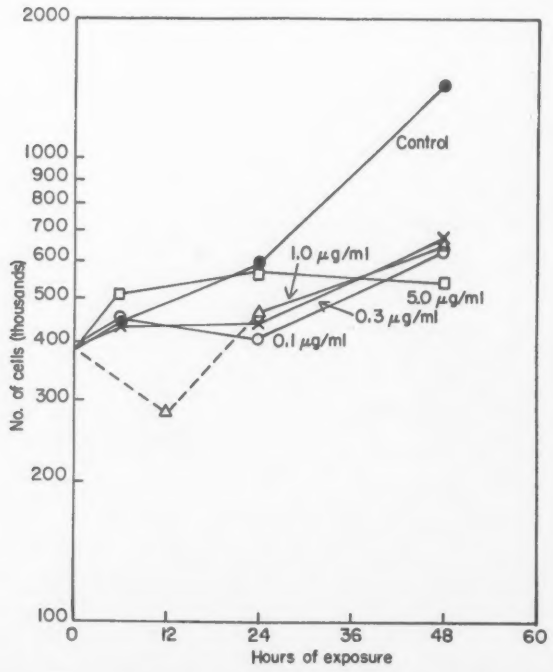

FIGURE 4.—Effects of NSC-135758 upon the proliferation of glass-attached cultured H.Ep. No. 2 cells.

indicates that this agent at these concentrations causes little interference with the progression of cells from G_1 into S phase or with their progression far enough into S phase to be scored as labeled cells.

Determinations of the viability of the H.Ep. No. 2 cells were made in parallel with the determinations of the effects upon progression of cells through the cell cycle. When cells were exposed to 0.1 μg/ml of NSC-135758, the survival was 30% after 6 hours, 2% after 24 hours, and 3% after 48 hours. At concentrations of 0.3, 1.0, and 5.0 μg/ml, all cells were nonviable after exposures of 6, 24, and 48 hours.

Effects on Adenocarcinoma 755 Cells

The curves of figure 7 show that the control cells were proliferating exponentially throughout the 48-hour period of the experiment with a doubling time of approximately 12 hours, but the rate of proliferation decreased as the concentration of NSC-135758 was progressively increased. The graphs of figure 8 show that enlargement of the cells occurred with all concentrations of the agent and all periods of exposure.

Experiments to determine the effects upon progression through the cell cycle and upon viability were not performed with Adenocarcinoma 755 cells.

DISCUSSION

The experimental results obtained with cultured L1210, H.Ep. No. 2, and Adenocarcinoma 755 cells are consistent in showing that inhibition of proliferation of the cells is accompanied by cell enlargement. Although cells initially in G_2 phase when exposure to the agent is begun can probably proceed through mitosis and divide, cells that are initially in G_1 and in S phases accumulate in the G_2 phase. There is probably little or no slowing of the progression of the cells through G_1 nor of their entry into S phase, but evidently progression through the S phase is slowed because the time required for the accumulation of cells with a G_2-phase content of DNA is greater than would be expected upon the basis of what is known about the lengths of the phases of the cycle (9–13). Decreased synthesis of DNA by cultured cells after periods of exposure to this agent has also been observed directly (14). It is not necessary for cells to proceed to G_2 during the period of exposure to the agent in order to kill the cells, because cells exposed for only 6 hours do not survive (fig 3, table 1).

Our results for experiments with H.Ep. No. 2 cells are similar to those obtained previously in similar experiments with mechlorethamine and two monofunctional nitrogen mustards (8). This similarity of

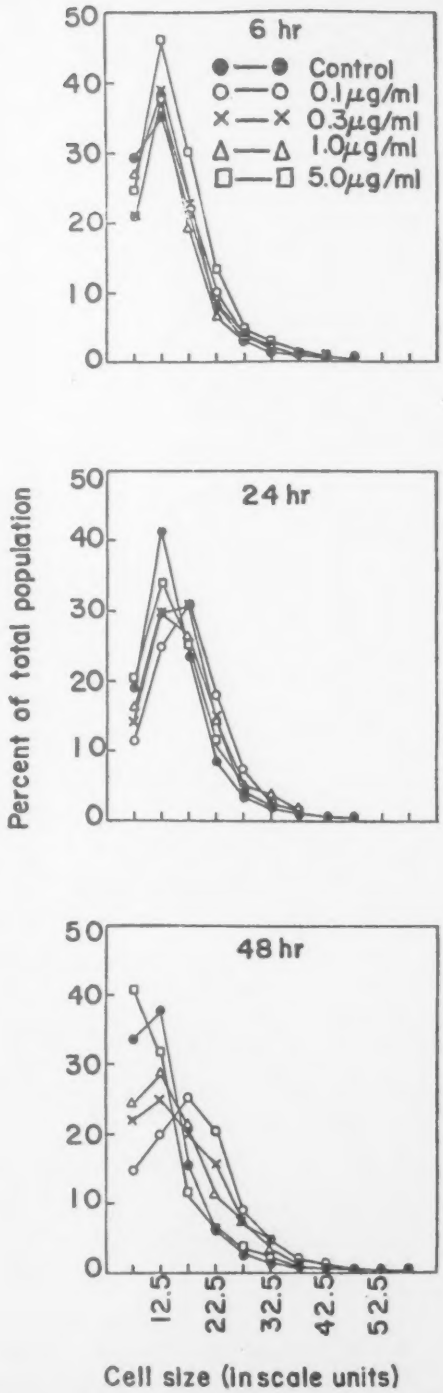

FIGURE 5.—Effects of NSC-135758 upon the size distribution of glass-attached cultured H.Ep. No. 2 cells.

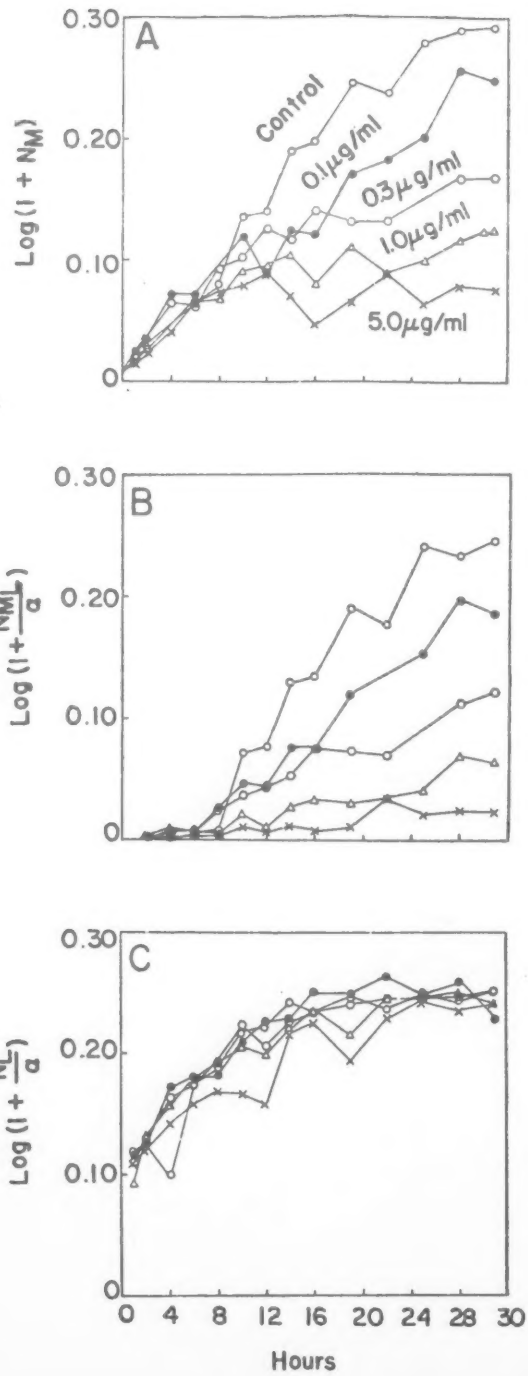

FIGURE 6.—Effects of NSC-135758 upon the progression of glass-attached cultured H.Ep. No. 2 cells through the cell cycle. A = the accumulation of mitotic cells (unlabeled plus labeled) during incubation in the presence of colcemid; B = the accumulation of labeled mitotic cells during incubation in the presence of colcemid; C = the accumulation of labeled cells (interphase plus mitotic) during incubation in the presence of colcemid. N_M, fraction of the population of cells that consists of mitotic cells; N_{M_L}, fraction of the population of cells that consists of labeled mitotic cells; N_L, fraction of the population of cells that consists of labeled cells (both mitotic and interphase); α, constant. *See* ref 8 for derivation and explanation of these accumulation functions.
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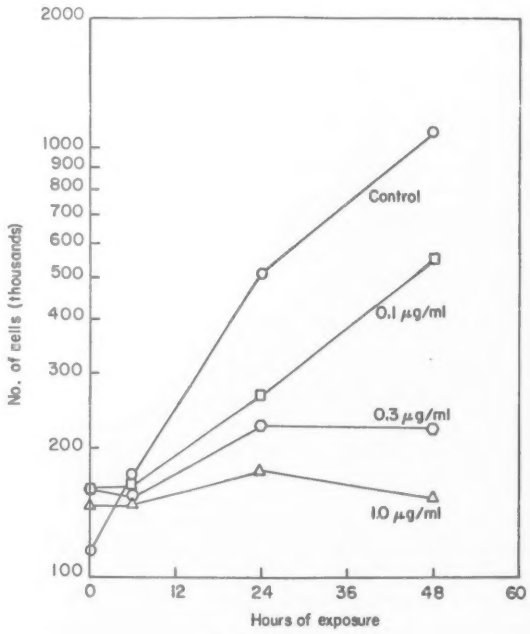

FIGURE 7.—Effects of NSC-135758 upon the proliferation of cultured Adenocarcinoma 755 cells.

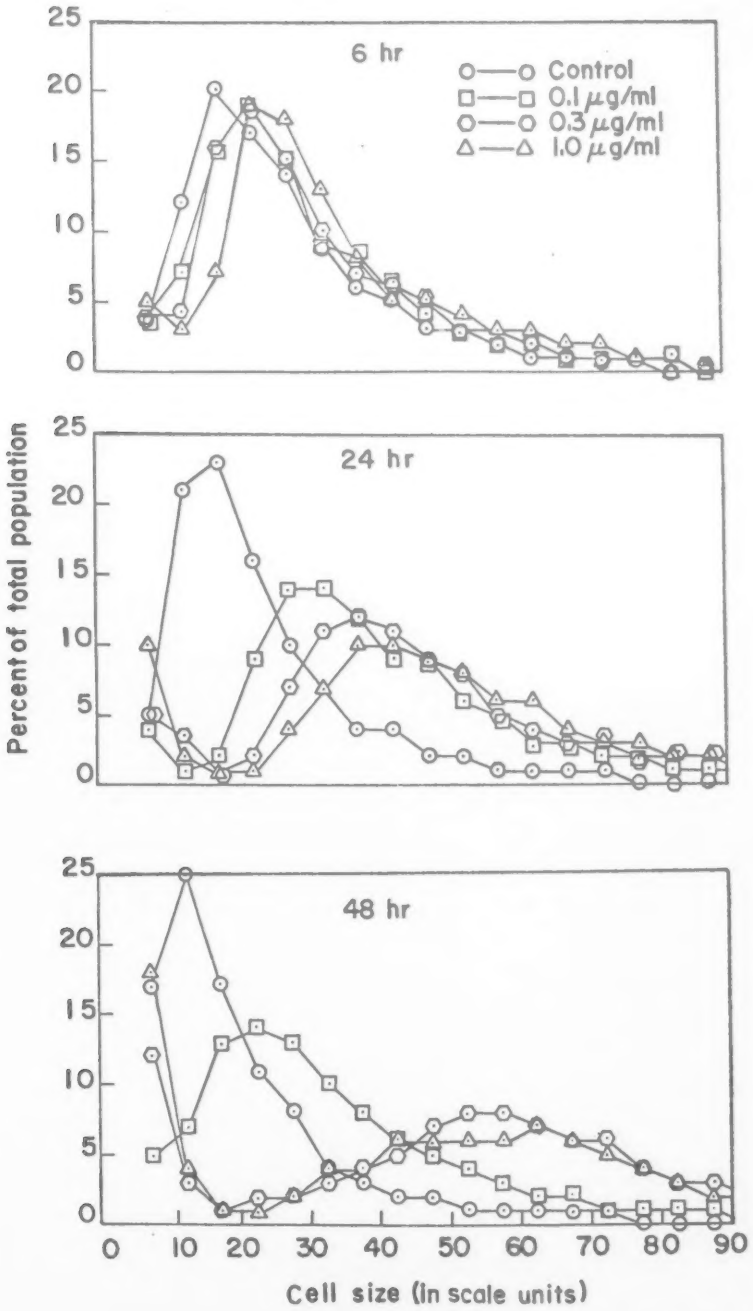

FIGURE 8.—Effects of NSC-135758 upon the size distribution of cultured Adenocarcinoma 755 cells.

effect is understandable, because NSC-135758 contains two 2-chloroethylamino groups and it has chemically demonstrable alkylating activity (3,14).

Rao and Mahagaokar (15) have also studied the effects of this agent upon cultured cells. They exposed cultured Chinese hamster ovary cells to 5 or 10 μg of NSC-135758 for 1 hour and then observed the rate of labeling with C^3H_3-deoxythymidine and the rate of accumulation of mitotic cells in the presence of colcemid. These investigators also concluded that the agent did not interfere with the entry of cells into S but delayed the entry of G_2 cells into mitosis. They observed chromatid aberrations for all of the cells that were in S phase and about half of the cells that were in G_2 phase at the time of exposure to the agent. Since similar chromatid breaks and exchanges were not detected in previous similar experiments with mechlorethamine, there are some differences in the effects of this agent and those of mechlorethamine.

It is of additional interest that lines of L1210 that were resistant to cyclophosphamide were sensitive to NSC-135758 (3,16) and to melphalan (16).

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Studies With 2,5-Piperazinedione, 3,6-Bis(5-chloro-2-piperidyl)-, Dihydrochloride. II. Effects on Macromolecular Synthesis in Cell Culture and Evidence for Alkylating Activity[1,2,3]

R. W. Brockman,* S. C. Shaddix, M. Williams, and R. F. Struck[4,5]

SUMMARY

2,5-Piperazinedione, 3,6-bis(5-chloro-2-piperidyl)-, dihydrochloride (NSC-135758) inhibited DNA synthesis but not RNA and protein synthesis in Adenocarcinoma 755 cells in culture. The expression of such inhibition was delayed in time; it was necessary to expose tumor cells to NSC-135758 for 10–12 hours before measuring the macromolecular synthesis in order to demonstrate selective inhibition of DNA synthesis. Inhibition of DNA synthesis was demonstrated to be irreversible in human epidermoid carcinoma cells in culture. Exposure of cells in suspension culture to NSC-135758 or to melphalan for 1–4 hours, and then incubation of cells in the absence of an inhibitor for 20 hours, resulted in preferential inhibition of DNA synthesis; inhibition of RNA synthesis was observed under these conditions but was less pronounced. Chemical evidence for alkylating activity of NSC-135758 and of the bis-aziridine derived from it (NSC-201424) was obtained by demonstrating their reaction with 4-(*p*-nitrobenzyl)pyridine. NSC-135758 was more potent as a cytotoxic agent than was its derivative, a result which suggests that NSC-135758 is the active alkylating agent. Reaction of NSC-135758 with diethylamine was examined; the product obtained upon alkylation of diethylamine by NSC-135758 was identified from its proton magnetic resonance spectrum and by field desorption mass spectral analysis. These results support the view that NSC-135758 acts as an alkylating agent in inhibiting DNA synthesis and cell proliferation of tumor cells in culture.

[Cancer Treat Rep 60:1317–1324, 1976]

The known biologic effects of NSC-135758 include: (*a*) inhibition of growth of human tumors in the chick embryo (1) and of transplantable mouse neoplasms in vivo (2,3); (*b*) irreversible inhibitory effects on proliferation and viability of tumor cells in culture and in the mouse (4,5); (*c*) prevention of the progression of tumor cells in culture through S phase to mitosis and accumulation of cells with G_2-phase DNA content (5); and (*d*) enlargement of tumor cells in culture and in vivo, ie, giant cell formation (5). Concurrent with the work described by Wheeler et al (5), we examined the effects of NSC-135758 on macromolecular synthesis in tumor cells in culture and gathered chemical evidence for the capacity of this compound to act as an alkylating agent.

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[2]Supported by contract N01-CM-43784 from the Division of Cancer Treatment (DCT), National Cancer Institute (NCI), National Institutes of Health (NIH), Department of Health, Education, and Welfare.

[3]2,5-Piperazinedione, 3,6-bis(5-chloro-2-piperidyl)-, dihydrochloride: NSC-135758; CAS reg. No. 41109-80-2.

[4]Biochemistry (Dr. Brockman, Mrs. Shaddix, and Miss Williams) and Organic Chemistry (Dr. Struck) Departments, Southern Research Institute, Birmingham, Ala.

[5]We wish to thank Miss Bonnie Bowdon and Mr. Marion Kirk for their assistance in the chemical studies of alkylating activity of NSC-135758, Miss Doris Adamson for her assistance in the biologic studies in cell culture, and Mr. Thomas Herren for quantitative determinations of radioactivity. We are also indebted to Dr. Charles Gitterman, Merck Sharp and Dohme Research Laboratories (Rahway, NJ), for valuable discussions of aspects of this work.

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MATERIALS AND METHODS

Compounds

Melphalan, NSC-135758 (6), and the bis-aziridine derived from NSC-135758 (NSC-201424) (7) were obtained from the Drug Development Branch, Drug Research and Development Program, DCT, NCI, NIH.[6] Radioactive compounds were purchased from the New England Nuclear Corporation (Cambridge, Mass): C^3H_3-thymidine (6.7 curies/mmol); 5-^{3}H-uridine (26.2 curies/mmol); 4,5-^{3}H-L-leucine (50 curies/mmol); and sodium ^{14}C-formate (3.0 millicuries/mmol). 4-(*p*-Nitrobenzyl)pyridine (NBP) was obtained from the Aldrich Chemical Company (Milwaukee, Wis).

Cell Lines

Adenocarcinoma 755 cells (Ca755) and human epidermoid carcinoma cells (H.Ep.-2) were grown in suspension culture in SRI-14 cell-culture medium (8). Flasks containing 30-ml suspensions of cells (30,000–50,000 cells/ml) were incubated overnight and cell counts were made with a model B Coulter Electronic Particle Counter at the time of initiation of experiments and during the period of examination of macromolecular synthesis.

Examination of the Effect of Agents on Macromolecular Synthesis

The agent under study was added to suspensions of cells, prepared as described above, followed by the addition of a radioactive substrate; the time interval between addition of inhibitor and introduction of labeled precursor was varied, as indicated in the figures and tables. In some experiments, cells in suspension culture were exposed to inhibitors for a given period of time and were then incubated in medium free of inhibitor.

Samples were taken from each flask for a cell count before the addition of an inhibitor and 6 hours after the addition of a radioactive precursor. After the addition of a radioactive substrate, 5-ml samples of cell suspensions (approximately 100,000 cells/ml) were removed from control and treated cell suspensions after intervals of 0.5, 1, 2, 4, and 6 hours; in some experiments the 0.5-hour sample was omitted. Cells were collected as a pellet by centrifugation and were washed three times in cold 0.9% saline by resuspending the cells and centrifuging them. The washed pellet of cells was dispersed in 2.5 ml of 0.1% sodium lauryl sulfate containing added carrier DNA to give a final concentration of 50 μg/ml of DNA.

One milliliter of cold 20% (wt/vol) trichloroacetic acid (TCA) was added to 1.0 ml of the sodium lauryl sulfate solution; the precipitate was collected by suction filtration on Whatman glass-fiber filters and the filters were washed thoroughly with 10% TCA and then with 80% aqueous ethanol for determination of TCA-insoluble radioactivity. The filters thus prepared were placed in 1.0 ml of Soluene (Packard Instrument Co, Downers Grove, Ill) and incubated overnight at room temperature; a toluene scintillator was added and quantitative determinations of radioactivity were made in a Packard Tri-Carb Liquid Scintillation Spectrometer.

To 1 ml of the initially prepared sodium lauryl sulfate solution of cells was added 4N NaOH (0.1 ml). The alkaline solution was incubated overnight at 37°C to hydrolyze the RNA. The solution was neutralized with 0.1 ml of 4 N HCl, and cold TCA was added to give a final concentration of 10% TCA. The precipitated DNA was collected on glass-fiber filters and prepared for quantitative determination of radioactivity as described above for examination of TCA-insoluble activity.

When C^3H_3-thymidine (2 microcuries/ml) was the radioactive substrate, the incorporation of radioactivity into TCA-insoluble material afforded a measure of the rate of DNA synthesis. When 5-^{3}H-uridine (2 microcuries/ml) was the radioactive substrate, the incorporation of radioactivity into the TCA-insoluble material gave a measure of the rate of RNA and DNA synthesis and radioactivity in the NaOH-stable TCA-insoluble fraction provided a measure of the rate of incorporation of 5-^{3}H-uridine (as deoxycytidylate) into DNA. The difference between these values, ie, TCA-insoluble activity (RNA + DNA) minus NaOH-stable TCA-insoluble activity (DNA), gave a measure of the rate of incorporation of 5-^{3}H-uridine into RNA. The addition of 4,5-^{3}H-L-leucine (2 microcuries/ml) to cell-culture suspensions and determination of radioactivity incorporated into the TCA-insoluble fraction provided a measure of protein synthesis.

Reaction of NSC-135758 With NBP

The extent of the reaction of NSC-135758 with NBP provided a measure of its chemical reactivity as an alkylating agent, according to the method described by Friedman and Boger (9). The procedure followed in these experiments was similar to that used by Wheeler and Chumley (10) except that NSC-135758, the bis-aziridine derived from NSC-135758,

[6]Melphalan: NSC-8806; CAS reg. No. 3223-07-2; alanine, 3-[*p*-[bis(2-chloroethyl)amino]phenyl]-, monohydrochloride, L-.

NSC-201424: CAS reg. No. 41944-72-3; 2,5-piperazinedione, 3,6-bis(1-azabicyclo[3.1.0]hex-2-yl)-.

and mechlorethamine,[7] a reference compound, were reacted with NBP in 0.05 M Tris buffer, pH 7.4, instead of in acetate buffer. The reaction mixture consisted of 0.4 ml of a 5% solution of NBP in acetone, 4 ml of Tris buffer, 0.2 μmol of mechlorethamine, or 1.0 μmol of test compound in Tris buffer. The reaction mixture was heated at 37°C for 2 hours or at 100°C for 30 minutes. Acetone (2 ml) was added, followed by ethyl acetate (5 ml) and then by 0.25 N NaOH (1.5 ml). This mixture was shaken vigorously by hand and then centrifuged briefly in a clinical centrifuge to separate the organic and aqueous phases. The organic layer was removed and the optical density was measured at 540 nm. A_{540} of test compound (1 μmol) was compared to A_{540} of 1 μmol of mechlorethamine (from a standard curve) to give the R_{HN2}.

Alkylation of Diethylamine by NSC-135758

NSC-135758 was allowed to react with excess diethylamine in acetone for 12 hours. Evaporation, trituration with acetone, and filtration removed some unchanged NSC-135758. Evaporation of the acetone filtrate to dryness in vacuo gave a product whose proton magnetic resonance (PMR) spectrum indicated alkylation of diethylamine by NSC-135758: PMR^{TMS}_{DMSO}:δ 1.2 (t, CH_3 of CH_3CH_2), 1.5–2.3 (m, $-CH_2-CH_2-$), 2.85 (d of d, CH_2 of CH_3CH_2), 3.3–4.6 (complex m, methinyl H of piperidyl and piperazinedione rings and $-CH_2-NH$), 3.2–6.8 (broad, NH), 8.2 (s, NH). Field desorption mass spectral analysis of the reaction product demonstrated the presence of a molecular ion peak (M^+ 422, no Cl) for the dialkylation product.

[7]Mechlorethamine: NSC-762; CAS reg. No. 55-86-7; diethylamine, 2,2′-dichloro-*N*-methyl-, hydrochloride; HN2.

RESULTS

DNA synthesis in Ca755 cells was inhibited by NSC-135758 when the cells in suspension culture were exposed to the agent for 10 hours prior to incubation with radioactive thymidine (fig 1). No inhibition of incorporation of ^{3}H-thymidine into DNA was observed when the radioactive substrate was introduced into the culture immediately after the inhibitor and DNA synthesis was followed over a 6-hour period (fig 1). NSC-135758 did not inhibit RNA and protein synthesis under conditions in which DNA synthesis was inhibited (fig 2). That is, pretreatment of Ca755 cells with

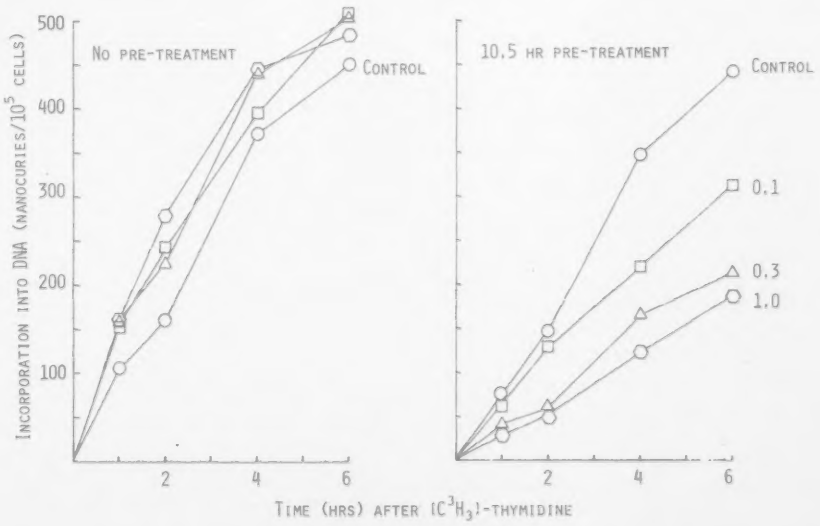


FIGURE 1.—Delayed inhibition (concentrations are given in μg/ml) of DNA synthesis in Ca755 cells in culture by NSC-135758. Cells in suspension culture were treated with NSC-135758 for 15 mins (no pretreatment) or for 10.5 hrs prior to the addition of C^3H_3-thymidine.

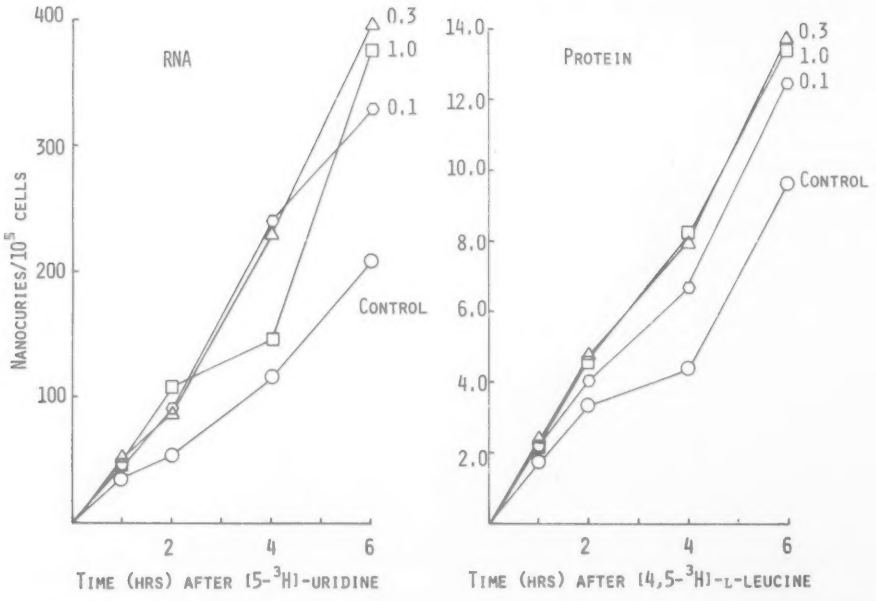


FIGURE 2.—Lack of inhibition of RNA and protein synthesis in Ca755 cells in culture by NSC-135758 under conditions in which DNA synthesis is inhibited. Cells in suspension culture were treated with NSC-135758 at the indicated concentration (μg/ml) for 10.5 hrs prior to the addition of radioactive substrates.

NSC-135758 for 10–12 hours before incubation with 5-^{3}H-uridine or 4,5-^{3}H-L-leucine did not result in inhibition of incorporation of these precursors into macromolecules. In fact, incorporation of these substrates into RNA and protein was greater in cells pretreated with NSC-135758 than in untreated control cells. ^{14}C-formate incorporation into DNA also was inhibited, as would be anticipated from results of the thymidine incorporation study; formate incorporation into RNA was not inhibited under these experimental conditions (fig 3). Results of these experiments (figs 1–3) indicate that inhibition of DNA synthesis by NSC-135758 was selective and that the onset of inhibition of DNA synthesis was delayed in time.

The irreversible nature of NSC-135758 inhibition of DNA synthesis was observed when H.Ep.-2 cells in suspension culture were exposed to NSC-135758 for a period of 4 hours and then the cells were incubated in medium free of inhibitor for 20 hours prior to measurement of DNA synthesis. Results summarized in figure 4 show that DNA synthesis was inhibited by > 80% at the higher concentrations of NSC-135758 (1.0 and 3.0 μg/ml). At these higher levels of NSC-135758, the incorporation of 5-^{3}H-uridine into RNA was inhibited by 30%.

Effects on nucleic acid synthesis of NSC-135758 and of a classic alkylating agent, melphalan, were compared in H.Ep.-2 cells growing in suspension culture. Cells were exposed to inhibitors for 1 hour and were then suspended in fresh medium free of inhibitor for 20 hours before measuring nucleic acid synthesis. DNA synthesis was inhibited in a dose-dependent manner by these agents (fig 5). Inhibition of RNA synthesis in H.Ep.-2 cells was also observed under these conditions and was more pronounced with melphalan than with NSC-135758 (fig 6).

NSC-135758 and NSC-201424 (fig 7) were compared for their effects on cell viability and on DNA synthesis in H.Ep.-2 cells (table 1). On a molar basis, NSC-135758 was the more effective inhibitor. The molar concentration of the bis-aziridine required for 50% inhibition of cell viability and DNA synthesis was approximately ten times that needed for NSC-135758.

A comparison of the alkylating activity of NSC-135758 and of NSC-201424 was made by observing

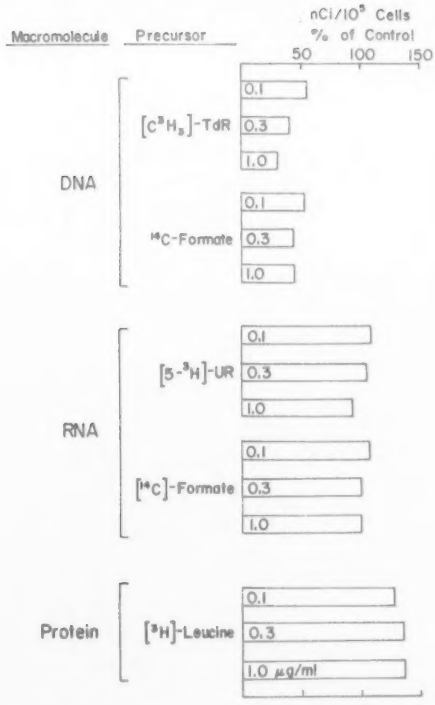


FIGURE 3.—Effect of NSC-135758 on macromolecular synthesis in Ca755 cells in culture. Cells in suspension culture were treated with inhibitor for 10.5 hrs before the addition of a radioactive precursor; the rate of macromolecular synthesis was then measured over a 6-hr period (as shown in figs 1 and 2). The results summarized here are based on the 6-hr sample.

the extent of reaction of these agents with NBP at pH 7.4 at 37°C and at 100°C (table 2). Reactivity of both compounds was low at 37°C; at 100°C these compounds were about equally reactive on a molar basis relative to the reactivity of mechlorethamine.

The PMR spectrum of the product of the reaction of NSC-135758 and diethylamine in acetone indicated that alkylation of diethylamine occurred (*see* Methods section). Field desorption mass spectral analysis of the reaction product indicated the presence of the dialkylation product (M^+422) obtained by replacement of the halogens of NSC-135758 by diethylamine moieties.

DISCUSSION

NSC-135758 is a potent inhibitor of DNA synthesis and of cell proliferation in Ca755 and in H.Ep.-2 cells in culture. In order to demonstrate inhibition of DNA synthesis in tumor cells in culture, it was necessary to treat the cells with inhibitor for 10–12 hours before examining the incorporation of tritiated thymidine into DNA (fig 1). Also, inhibition of DNA synthesis was selective under these conditions; that is, RNA and protein synthesis were not inhibited in Ca755 cells after exposure to the inhibitor for 10 hours (fig 2). The observed increased incorporation of precursors into RNA and protein of Ca755 cells is probably a result of unbalanced growth and consequent cell

TABLE 1.—Cytotoxic effects and inhibition of DNA synthesis produced in H.Ep.-2 cells by NSC-135758 and NSC-201424

	Concentration for 50% inhibition			
	Cell viability†		DNA synthesis‡	
Compound*	μg/ml	μM	μg/ml	μM
NSC-135758	0.11	0.26	0.25§	0.59
NSC-201424	1.05	3.8	1.8	6.5

*Molecular weight is 422 for NSC-135758 and 276 for NSC-201424 (*see* fig 7); the bis-aziridine derived from NSC-135758 was described by Jensen et al (7).

†H.Ep.-2 cells in suspension culture were exposed to a range of concentrations of inhibitors for 3 hrs and then cloned in medium free of inhibitor (5).

‡Suspension cultures of H.Ep.-2 cells were exposed to a range of concentrations of inhibitors for 11.5 hrs; methyl-^{3}H-thymidine was added and DNA synthesis was followed over a 6-hr period.

§Concentration of NSC-135758 that produced 50% inhibition of DNA synthesis was 0.3 μg/ml when cells were exposed to NSC-135758 for 4 hrs and then incubated in its absence for 20 hrs before introduction of ^{3}H-thymidine (*see* fig 4).

enlargement. This phenomenon was observed by Wheeler et al (5) in NSC-135758-inhibited Ca755 and H.Ep.-2 cells. Cell enlargement was also observed in L1210 ascites tumor cells in mice treated with NSC-135758 in vivo. The observed delayed inhibition of DNA synthesis can be understood on the basis of the observation that NSC-135758 arrested L1210 cells in culture in G_2 phase of the cell cycle (5). This being the case, those cells in the population that were in mitosis or in G_1 phase at the time of treatment would progress through S phase but, as cells accumulated in G_2, DNA synthesis would decrease.

Inhibition of DNA synthesis was found to be irreversible in tumor cells in culture. Thus, exposure of H.Ep.-2 cells to NSC-135758 for 4 hours followed by incubation of the cells in inhibitor-free medium resulted in inhibition of DNA synthesis 20 hours later (fig 4). At low concentrations of NSC-135758 (0.03 and 0.1 μg/ml) the rate of incorporation of 5-^{3}H-uridine into RNA was greater than that in untreated controls; higher concentrations of inhibitor resulted in partial inhibition of RNA synthesis.

A direct comparison of effects of NSC-135758 and of melphalan on nucleic acid synthesis in H.Ep.-2 cells in culture was made. Both compounds inhibited DNA synthesis but inhibition of RNA synthesis by melphalan was more pronounced than it was with NSC-135758. It is of interest that similar results were obtained in

TABLE 2.—Alkylating activity of NSC-135758 and NSC-201424

Compound†	R_{HN2}*	
	100°C	37°C
NSC-135758	0.18, 0.22	0.007, 0.02
NSC-201424	0.22, 0.26	0, 0.001

*Reactivity with NBP is expressed relative to the reactivity of mechlorethamine with NBP as unity; comparisons were made on a molar basis at the temperature indicated (*see* Materials and Methods).

†*See* fig 7.

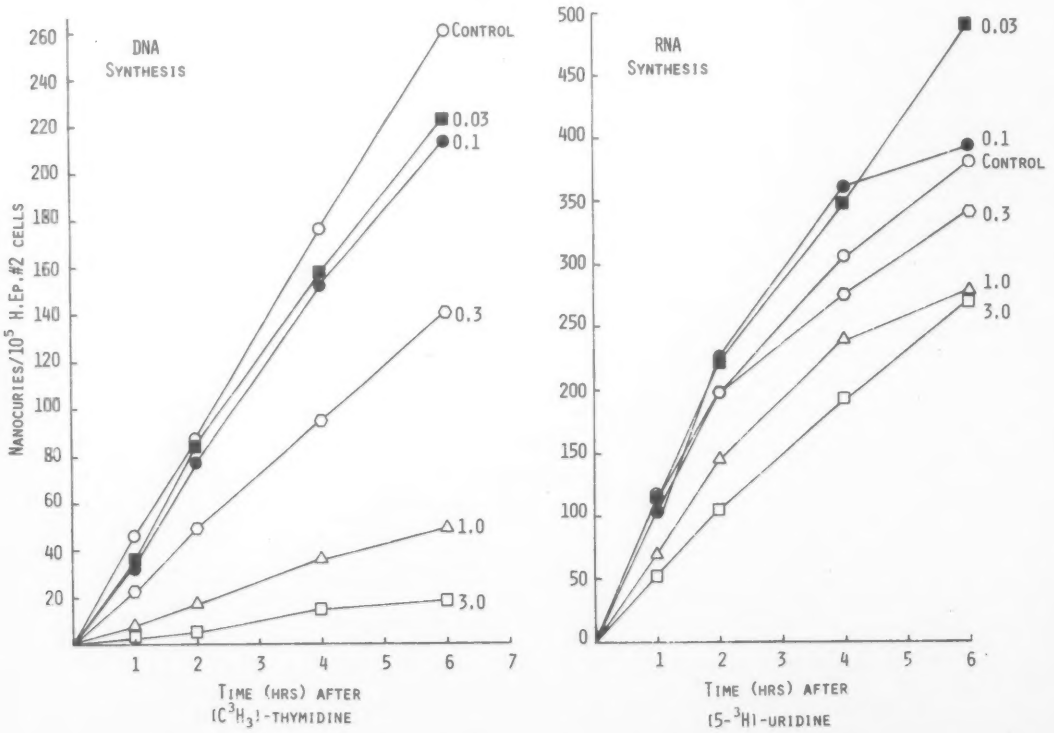


FIGURE 4.—Irreversible effect of NSC-135758 on nucleic acid synthesis in H.Ep.-2 cells in suspension culture. Cells were exposed to the inhibitor at the concentrations indicated for 4 hrs and then were aseptically removed from inhibitor-containing medium by centrifugation. After incubation for 20 hrs in inhibitor-free medium, radioactive substrates were added and DNA and RNA synthesis were measured.

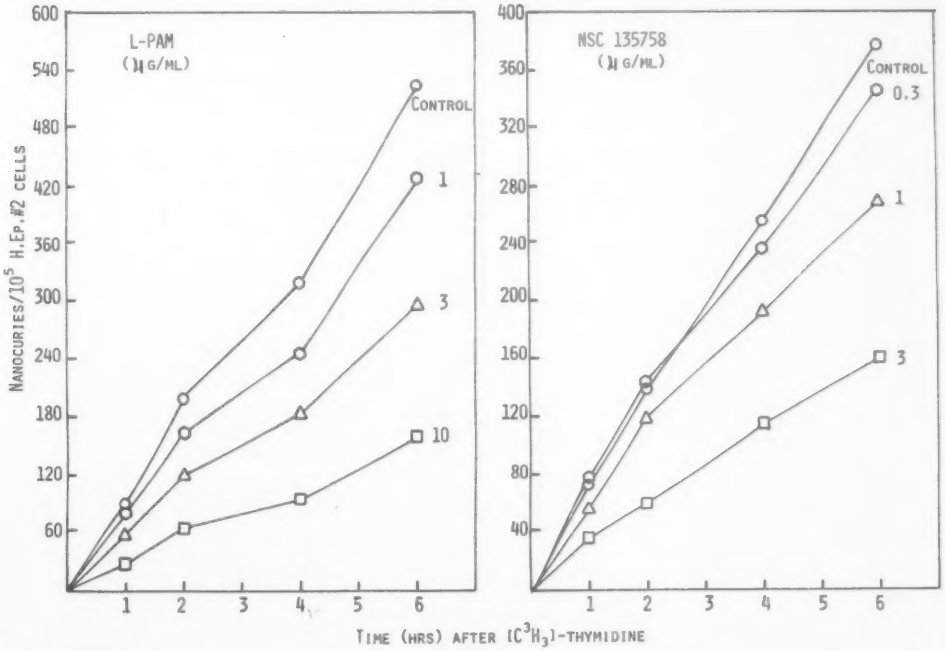


FIGURE 5.—Inhibition of DNA synthesis in H.Ep.-2 cells by melphalan and NSC-135758. Cells growing in suspension culture were exposed to the inhibitors for 1 hr; H.Ep.-2 cells were then suspended in fresh medium free of inhibitor and nucleic acid synthesis was examined 20 hrs later by incubating the cells with methyl-³H-thymidine.

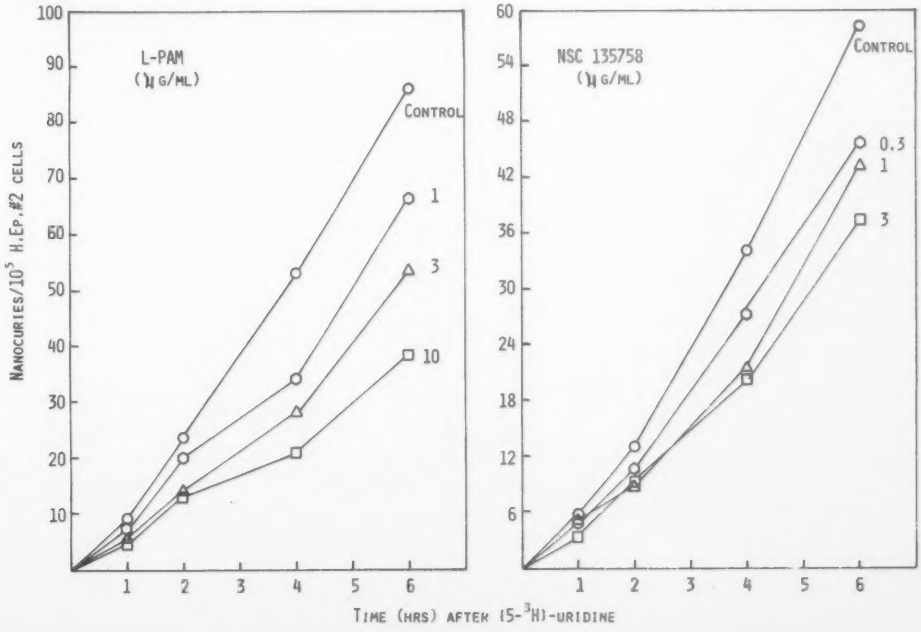
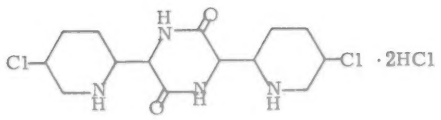


FIGURE 6.—Effect of melphalan and NSC-135758 on the incorporation of 5-³H-uridine into RNA of H.Ep.-2 cells in suspension culture (*see* fig 5).



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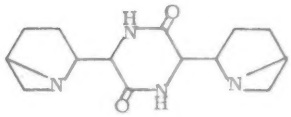

II

FIGURE 7.—Structure of NSC-135758(I) and its derivative NSC-201424(II).

L1210 cells treated with these agents in vivo (3). Both melphalan and NSC-135758 inhibited DNA synthesis in L1210 cells, but only melphalan inhibited RNA synthesis.

Chemical evidence for the alkylating activity of NSC-135758 was demonstrated by its reaction with NBP and with diethylamine. It was considered possible that NSC-135758 might form the corresponding bis-aziridine, NSC-201424 (fig 7), which could be the active alkylating agent. We compared NSC-135758 and NSC-201424 (7) and found that NSC-135758 was more potent as an inhibitor of DNA synthesis. Effects of the two compounds on cell viability paralleled their potency as inhibitors of DNA synthesis (table 1). These results suggest that NSC-135758 is the active alkylating agent. The bis-aziridine derivative is of interest since its uptake by cells, tissue distribution, and other pharmacologic properties might be different from those of NSC-135758.

Chemotherapeutic effects of NSC-135758 on sensitive and resistant neoplasms were examined by Schabel et al (3). It is of considerable interest that sublines of L1210 leukemia resistant to BCNU and to cyclophosphamide were sensitive to NSC-135758. Similarly, melphalan was chemotherapeutically active against these same resistant neoplasms (3). Recently, a subline of L1210 resistant to melphalan has been isolated in these laboratories* and it has proven to be sensitive to NSC-135758. More detailed studies of the biochemical mechanism of action of NSC-135758 should prove interesting.

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Studies With 2,5-Piperazinedione, 3,6-Bis(5-chloro-2-piperidyl)-, Dihydrochloride. III. Biochemical and Therapeutic Effects in L1210 Leukemias Sensitive and Resistant to Alkylating Agents: Comparison With Melphalan, Cyclophosphamide, and BCNU[1,2,3]

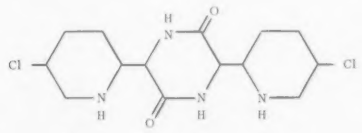
F. M. Schabel, Jr,* M. W. Trader, W. R. Laster, Jr, S. C. Shaddix, and R. W. Brockman[4]

SUMMARY

2,5-Piperazinedione, 3,6-bis(5-chloro-2-piperidyl)-, dihydrochloride (NSC-135758) selectively inhibits DNA synthesis in L1210/0 leukemia and in cyclophosphamide-resistant L1210 (L1210/CPA). Melphalan (L-PAM) inhibits nucleic-acid synthesis but not protein synthesis in L1210/0 and L1210/CPA. CPA selectively inhibits DNA synthesis in L1210/0 but does not inhibit DNA synthesis in L1210/CPA. NSC-135758 is as active in vivo against L1210/CPA and BCNU-resistant L1210 (L1210/BCNU) as it is against L1210/0. It is inactive against ic implanted L1210/0. These data clearly indicate important differences in the biologic activity of this compound compared to either CPA or BCNU. L-PAM is similar to NSC-135758 in activity against L1210/CPA and L1210/BCNU.

[Cancer Treat Rep 60:1325–1333, 1976]

2,5-Piperazinedione, 3,6-bis(5-chloro-2-piperidyl)-, dihydrochloride (NSC-135758) was isolated from fermentation broths of *Streptomyces griseoluteus* by Gitterman et al (1). The structure was deduced from chemical and physical evidence by Arison and Beck (2); confirmation of this structure was obtained by means of field desorption mass spectrometry (3).



Results of our studies with NSC-135758 (4,5), in agreement with those of Gitterman et al (1), indicated that this compound probably exerts its inhibitory effect on the growth of tumor cells through its capacity to act as an alkylating agent. Irreversible effects of NSC-135758 on DNA synthesis, on cell proliferation, and on cell viability were observed in tumor cells in culture (4,5). NSC-135758 prevented tumor cells in culture from progressing through S phase to mitosis, and evidence was obtained that this agent produced an accumulation of cells with G_2-phase DNA content; giant cell formation also was observed (4,5).

In view of observations that NSC-135758 and melphalan (L-PAM) have similar inhibitory effects on nucleic-acid synthesis in human epidermoid (H.Ep.-2) cells in culture (5) and that L-PAM was an effective therapeutic agent against cyclophosphamide-resistant L1210 (L1210/CPA) and BCNU-resistant L1210 (L1210/BCNU) (6), we undertook a comparison of the effect of NSC-135758 on macromolecular synthesis in the parent L1210/0 and in L1210/CPA. Our results showed that NSC-135758 was an effective inhibitor of DNA synthesis in L1210/CPA. We therefore examined the therapeutic effectiveness of NSC-135758 against L1210/CPA and L1210/BCNU.

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[2]Supported by contracts N01-CM-43756 and N01-CM-43784 from the Division of Cancer Treatment (DCT), National Cancer Institute (NCI), National Institutes of Health, Department of Health, Education, and Welfare.

[3]Drugs used in this study were supplied by Drug Research and Development Program, DCT, NCI.

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MATERIALS AND METHODS

Biochemical Studies

The following radioactive compounds were obtained from the New England Nuclear Corporation (Cambridge, Mass): methyl-^{3}H-thymidine (6.7 curies/mmol); 5-^{3}H-uridine (29.0 curies/mmol); and L-4,5-^{3}H-leucine (38.6 curies/mmol).

For a study of the effects of CPA, L-PAM, and NSC-135758 on macromolecular synthesis in intact L1210 cells, BDF_1 mice were implanted ip with 10^5 L1210/0 or L1210/CPA cells. On the sixth day after implant, animals were randomized and treated ip with a single dose of each agent equal to that which can be given each day on an every day, 1–9-day schedule without exceeding the LD10. These doses were 26.0 mg/kg of CPA, 4.3 mg/kg of L-PAM, and 2.2 mg/kg of NSC-135758. Twenty-four hours after drug treatment, the mice were killed, the ascitic fluid was removed with a syringe, and the residual tumor cells were collected by washing the peritoneal cavity with physiologic saline. The pooled tumor cells were centrifuged and rewashed with physiologic saline. Erythrocytes were lysed by a Tris-buffered isotonic ammonium chloride wash (7) and washed again with physiologic saline.

The washing procedures were done with cold solutions. Cells were separated from wash solutions by centrifugation in an international clinical centrifuge, suspended in Fischer's cell culture medium (8), and counted with a Coulter counter. Cell suspensions were diluted with Fischer's medium to concentrations of approximately 250,000 cells/ml and brought to 37°C in a water bath. Methyl-^{3}H-thymidine, 5-^{3}H-uridine, or L-4,5-^{3}H-leucine was added to suspensions of untreated control cells and to cells from animals treated in vivo with alkylating agents.

Samples were taken at 15-minute intervals over a 90-minute period for analysis of macromolecular synthesis. The general procedure for examining macromolecular synthesis in tumor cells has been described by Brockman et al (9) and is not presented here in detail. Illustrative results are presented in figure 1; complete results are summarized in table 1.

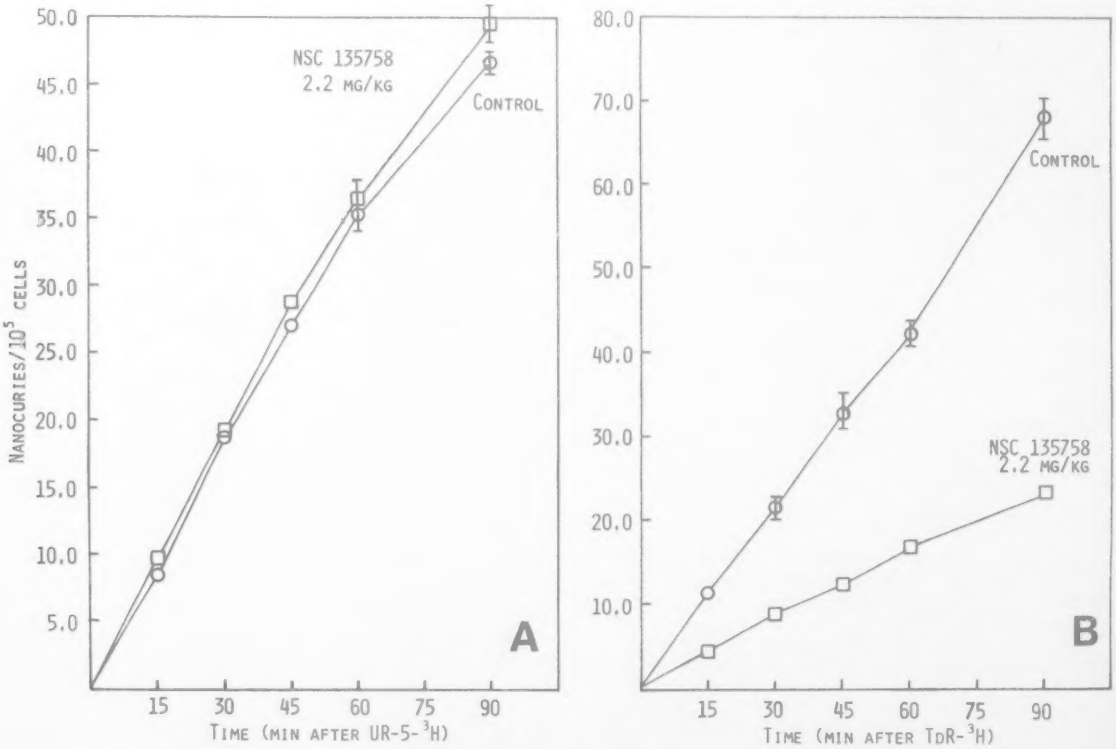

FIGURE 1.—A. Effect of NSC-135758 treatment in vivo on subsequent incorporation in vitro of 5-^{3}H-uridine into RNA in L1210/CPA cells. B. Inhibition of DNA synthesis in L1210/CPA cells by NSC-135758.

TABLE 1.—Comparison of the effects of NSC-135758, L-PAM, and CPA on macromolecular synthesis in sensitive (L1210/0) and resistant (L1210/CPA) L1210 leukemia

Leukemia* line	Inhibitor	Dose in vivo† (mg/kg)	Treated as % of control‡ (nanocuries/10^5 cells) DNA TdR-^{3}H	RNA UR-5-^{3}H	Protein Leu-4,5-^{3}H
L1210/0	NSC-135758	2.2	64 (±4)	108 (±15)	137 (±10)
L1210/0	L-PAM	4.3	42 (±4)	48 (±12)	110 (±23)
L1210/0	CPA	26.0	54 (±5)	100 (±2)	103 (±3)
L1210/CPA	NSC-135758	2.2	37 (±3)	106 (±2)	—
L1210/CPA	L-PAM	4.3	23 (±6)	43 (±11)	112 (±8)
L1210/CPA	CPA	26.0	236 (±3)	165 (±2)	215 (±8)

*L1210 cells (10^5) were implanted ip; on the 6th day after implant, mice were treated ip with the indicated dose of alkylating agent.

†The dose of each agent is the LD10 for a multiple-dose schedule (qd, 1–9 days).

‡Twenty-four hours after treatment of leukemia-bearing mice, tumor cells were harvested, washed, suspended in cell culture medium, and incubated with radioactive substrates in vitro. Samples were taken at 15-min intervals over a period of 90 mins; data presented are mean values (± standard deviation) for the rate of incorporation of radioactive precursors into macromolecules. TdR-^{3}H = methyl-^{3}H-thymidine; UR-5-^{3}H = 5-^{3}H-uridine; and Leu-4,5-^{3}H = L-4,5-^{3}H-leucine.

Sublines of L1210 Resistant to Alkylating Agents

L1210/CPA

The CPA-resistant subline of L1210 used was obtained from Dr. W. D. DeWys, who developed and described it (10). This subline has very marked (essentially complete) resistance to treatment with CPA in vivo, but retains sensitivity to BCNU (6), CCNU, and methyl-CCNU (table 2). It is also as sensitive to L-PAM as is the parent drug-sensitive L1210/0 (6) (table 2).

L1210/BCNU

The development of L1210/BCNU has been described (6). Ascites from this BCNU-resistant subline has been kept in frozen storage and removed as needed. It was maintained untreated when in serial passage, and periodic checks for continued drug resistance were made. Results of a recent test at the 75th passage, 15 weekly transfers after removal from frozen storage, are included in table 3.

L1210/BCNU is cross-resistant to CCNU and methyl-CCNU (unpublished data) and is as sensitive as L1210/0 to CPA and L-PAM (6).

Studies have been done on the comparative sensitivity of L1210/0 and L1210/CPA to CPA and NSC-135758 (table 4) and of L1210/0 and L1210/BCNU to BCNU and NSC-135758 (table 5).

The activity of NSC-135758 against ic implanted L1210/0 was also studied. BDF_1 mice were implanted ic with 10^4 L1210 cells and treated with NSC-135758 on single-dose or multiple daily dose (qd, 1–9 days) schedules. CCNU on a single-dose schedule was included as a known positive control. Collected data from two separate experiments are shown in table 6.

RESULTS AND DISCUSSION

Effect on Macromolecular Synthesis

NSC-135758, L-PAM, and CPA inhibited DNA synthesis in L1210/0 cells; none of these agents inhibited protein synthesis (table 1). However, L-PAM inhibited RNA synthesis in L1210/0 and in L1210/CPA, whereas NSC-135758 and CPA did not. It is of particular interest that both NSC-135758 and L-PAM inhibited DNA synthesis in L1210/CPA. CPA did not inhibit macromolecular synthesis in L1210/CPA; in fact, the rate of incorporation of radioactive substrates into DNA, RNA, and protein was significantly higher in CPA-treated L1210/CPA than in untreated controls. The basis for this increased incorporation of precursors into macromolecules in treated L1210/CPA cells is not yet understood (5).

Chemotherapy Studies

Studies on the in vivo activity of NSC-135758 indicate that it has moderate but clearly reproducible therapeutic activity against L1210/0. Only limited dose-schedule studies have been done, but the available data (table 4) indicate that equitoxic single-dose treatment is equal to multiple-dose treatment as determined by increased lifespan (ILS), but superior to multiple-dose treatment as indicated by the number of viable tumor cells alive at the end of therapy. This is generally consistent with the activity of CPA or L-PAM against L1210/0. However,

TABLE 2.—Comparative response of L1210/0 and L1210/CPA to treatment with L-PAM, BCNU, CCNU, and methyl-CCNU

Treatment: ip Day 1 only*			L1210/0					L1210/CPA			
Agent	Dosage (mg/kg/dose)	In terms of LD10	Concurrent toxicity‡ survivors/ total	Site and size of implant	% ILS (excluding "cures")	"Cures" (No./ total)	Approximate No. of cells alive at end of treatment†	Concurrent toxicity‡ survivors/ total	% ILS (excluding "cures")	"Cures" (No./ total)	Approximate No. of cells alive at end of treatment†
CPA	312	1.0	9/10	Ip; 10^7	+107	0/10	44	9/10	0	0/10	5.3×10^7
	208	0.67	10/10		+142	0/10	ca 1	10/10	0	0/10	5.3×10^7
	137	0.44	10/10		+92	0/10	296	10/10	−7	0/10	5.3×10^7
	104	0.33	10/10		+71	0/10	5.1×10^3	10/10	−13	0/10	5.3×10^7
	312	1.0	9/10	Ip; 10^6	+100	4/10	ca 1 (0.9)	9/10	0	0/7	5.3×10^6
	208	0.67	10/10		+89	1/10	ca 1 (2)	10/10	0	0/7	5.3×10^6
	200	0.64	10/10		+105	6/10	ca 1 (0.5)	10/10	0	0/10	5.3×10^6
	137	0.44	10/10		+84	0/10	ca 1	10/10	0	0/7	5.3×10^6
	104	0.33	10/10		+42	0/10	3.4×10^3	10/10	0	0/7	5.3×10^6
	312	1.0	9/10	Ip; 10^5	+120	5/10	ca 1 (0.7)	9/10	+8	0/9	1.9×10^4
	208	0.67	10/10		+110	1/10	ca 1 (2)	10/10	0	0/9	5.3×10^5
	137	0.44	10/10		+40	1/10	338 (2)	10/10	−9	0/9	5.3×10^5
	104	0.33	10/10		+30	0/10	2.3×10^3	10/10	−9	0/9	5.3×10^5
L-PAM	20	1.1	5/10	Ip; 10^7	+14	0/10	Toxic	9/10	+81	0/10	1.0×10^3
	13	0.68	10/10		+135	0/10	ca 1	10/10	+68	0/10	5.3×10^3
	9	0.47	10/10		+164	0/10	1.3×10^4	10/10	+62	1/10	1.2×10^3 (2)
	6	0.32	10/10		+71	0/10	5.1×10^3	9/10	+43	0/10	1.5×10^5
	20	1.1	5/10	Ip; 10^6	−11	0/10	Toxic	9/10	+72	1/7	100 (2)
	13	0.68	10/10		+78	0/10	4	10/10	+55	0/7	1.2×10^3
	9	0.47	10/10		+68	0/10	30	10/10	+55	0/7	1.2×10^3
	6	0.32	10/10		+47	0/10	1.3×10^3	9/10	+44	0/7	6.6×10^3
	20	1.1	5/10	Ip; 10^5	0	7/10	Toxic	9/10	+41	2/9	123 (2)
	13	0.68	10/10		+85	4/10	ca 1 (0.9)	10/10	+25	2/9	3.5×10^3 (2)
	9	0.47	10/10		+60	1/10	8 (2)	10/10	+41	1/9	123 (2)
	6	0.32	10/10		+45	0/10	131	9/10	+20	1/9	8.1×10^3 (2)
BCNU	40	1.0	8/10	Ip; 10^7	+335	4/10	Toxic	10/10	+150	7/10	ca 1 (0.4)
	27	0.68	10/10		+157	1/10	ca 1 (2)	10/10	+175	7/10	ca 1 (0.4)
	18	0.45	10/10		+114	0/10	17	10/10	+125	2/10	3 (2)
	13	0.33	10/10		+71	0/10	5.1×10^3	10/10	+75	0/10	2.3×10^3
	40	1.0	8/10	Ip; 10^6	+121	9/10	Toxic	10/10	—	7/7	— (< 0.1)
	27	0.68	10/10		+110	3/10	ca 1 (1)	10/10	+177	6/7	ca 1 (0.1)
	18	0.45	10/10		+57	1/10	197 (2)	10/10	+88	0/7	8
	13	0.33	10/10		+57	0/10	197	10/10	+66	0/7	231
	40	1.0	8/10	Ip; 10^5	+10	6/10	Toxic	10/10	−13	7/9	5.3×10^3 (0.2)
	27	0.68	10/10		+130	8/10	ca 1 (0.2)	10/10	+225	8/9	ca 1 (0.1)
	18	0.45	10/10		+75	2/10	ca 1 (2)	10/10	+62	5/9	2 (0.5)
	13	0.33	10/10		+50	0/10	51	10/10	+50	0/9	23
CCNU	60	1.2	2/10	Iv; 10^7	+262	0/10	Toxic	2/10	+100	1/10	Toxic
	30	0.60	—		+325	1/10	ca 1 (2)	—	+200	1/10	18 (2)
	15	0.37	—		+100	0/10	5.4×10^5	—	+80	0/10	3.6×10^5
	6	0.12	—		+37	0/10	2.1×10^7	—	+20	0/10	5.2×10^7
Methyl-CCNU	40	1.0	10/10	Iv; 10^7	+437	2/10	ca 1 (2)	10/10	+340	5/10	ca 1 (0.7)
	20	0.50	10/10		+250	0/10	142	10/10	+100	0/10	1.0×10^5
	10	0.25	—		+62	0/10	5.0×10^6	—	+50	0/10	2.2×10^7
	4	0.10	—		+25	0/10	4.0×10^7	—	0	0/10	ca 1.0×10^7

*Treatment with CCNU and methyl-CCNU on day 2 only.
†Based on median day of death (dying). Figures in parentheses are based on % survivors.
‡Non-tumor-bearing (normal) mice.

TABLE 3.—Comparison of the sensitivity of L1210/0 and L1210/BCNU to BCNU at the 12th and 75th passage of the BCNU-resistant subline

Tumor cell implant			Treatment			60th-day survivors/ total	Median		Approximate cell kill (logs)	Approximate No. of cells alive at end of treatment
Tumor	Cells	Route	Agent	Dosage (mg/kg/dose)	Route and schedule		Day of death (dying)	% ILS (excluding "cures")		
L1210/0	10^5	Ip	Control			0/20	8.0			
			BCNU	25	Ip; Day 1 only	8/10	8.5	+6	6	0.2*
				18		8/10	14.5	+81	6	0.2*
				25	Oral; Day 1 only	8/10	29.5	+268	6	0.2*
				18		6/10	19.0	+137	6	0.5*
L1210/BCNU (passage No. 12)	10^6	Ip	Control			0/20	8.0			
			BCNU	25	Ip; Day 1 only	0/10	10.0	+25	2	1.7×10^5†
				18		0/10	9.0	+12	1	1.0×10^6†
				25	Oral; Day 1 only	0/10	8.0	0	0	5.9×10^6†
				18		0/10	8.0	0	0	5.9×10^5†
L1210/0	10^5	Ip	Control			0/20	9.5			
			BCNU	40	Ip; Day 2 only	10/10	—	—	≥ 6	< 0.1*
				30		7/10	42.0	+342	6	0.4*
L1210/BCNU (passage No. 75)	10^5	Ip	Control			0/20	9.5			
			BCNU	40	Ip; Day 2 only	0/10	14.0	+47	4	599†
				30		0/10	12.0	+26	2	5.0×10^4†

*Based on 60th-day survivors ("cures").
†Based on median lifespan of dying animals only.

TABLE 4.—Comparative sensitivity of L1210/0 and L1210/CPA to CPA and NSC-135758

Treatment: ip			Concurrent toxicity* survivors/ total	Site and size of implant	L1210/0			L1210/CPA		
Agent	Dosage (mg/kg/dose)	Schedule			% ILS (excluding "cures")	"Cures" (No./ total)	Approximate No. of cells alive at end of treatment†	% ILS (excluding "cures")	"Cures" (No./ total)	Approximate No. of cells alive at end of treatment†
CPA	300	Day 2 only	10/10	Ip; 10^6	+187	3/10	ca 1 (1)	+12	0/10	4.2×10^6
	200		10/10		+112	0/10	21	+12	0/10	4.2×10^6
	130		10/10		+62	0/10	1.0×10^4	0	0/10	1.7×10^7
	30	qd, 2–10 days	8/10	Ip; 10^6	+31	0/10	Toxic	+12	0/10	Toxic
	20		9/10		+18	0/10	ca 1.0×10^9	+12	0/10	ca 1.0×10^9
	13		9/10		+12	0/10	ca 1.0×10^9	+12	0/10	ca 1.0×10^9
NSC-135758	10	Day 2 only	7/9	Ip; 10^6	+68	0/10	Toxic	+112	0/10	Toxic
	6.6		10/10		+68	0/10	4.6×10^3	+100	0/10	192
	4.4		9/9		+62	0/10	1.0×10^4	+62	0/10	1.4×10^4
	3.0	qd, 2–10 days	2/10	Ip; 10^6	+112	0/10	Toxic	+106	0/10	Toxic
	2.0		10/10		+100	0/10	2.2×10^7	+75	0/10	3.0×10^8
	1.3		10/10		+62	0/10	ca 9.0×10^8	+50	0/10	ca 9.0×10^8
CPA	300	Day 2 only	10/10	Ip; 10^5	+120	9/10	ca 1 (0.1)	+10	0/10	4.2×10^5
	300		10/10		+155	8/10	ca 1 (0.2)	+10	0/10	4.9×10^5
	200		10/10		+110	3/10	ca 1 (1)	0	0/10	1.7×10^6
	200		10/10		+77	1/10	102 (2)	+5	0/10	1.1×10^6
	130		10/10		+50	1/10	1.0×10^3(2)	0	0/10	1.7×10^6
	30	qd, 2–10 days	8/10	Ip; 10^5	+35	0/10	Toxic	0	0/10	Toxic
	20		9/10		+20	0/10	ca 9.0×10^8	0	0/10	ca 1.0×10^9
	13		9/10		+10	0/10	ca 9.0×10^8	+5	0/10	ca 1.0×10^9
NSC-135758	10	Day 2 only	7/9	Ip; 10^5	+90	6/9	Toxic	+90	3/10	Toxic
	9.7		10/10		+55	0/10	1.6×10^3	+75	0/10	16
	6.6		10/10		+80	1/10	10 (2)	+65	0/10	163
	6.5		10/10		+83	2/10	51 (2)	+25	0/10	4.5×10^4
	4.4		9/9		+55	0/10	464	+50	0/10	1.4×10^3
	3.0	qd, 2–10 days	2/10	Ip; 10^5	+110	0/10	Toxic	+25	0/10	Toxic
	2.6		10/10		+105	0/10	1.9×10^5	+95	0/10	2.2×10^5
	2.2		10/10		+22	0/10	2.5×10^4	+80	0/10	2.4×10^6
	2.0		10/10		+90	0/10	4.6×10^5	+50	1/10	1.3×10^8(2)
	1.5		9/10		+77	0/10	6.2×10^6	+40	0/10	2.9×10^8
	1.3		10/10		+60	0/10	4.6×10^7	+35	0/10	ca 9.0×10^8
	1.0		10/10		+44	0/10	3.9×10^8	—	—	—

*Non-tumor-bearing (normal) mice.
†Based on median day of death of dying animals. Figures in parentheses are based on % survivors.

TABLE 5.—Comparative sensitivity of L1210/0 and L1210/BCNU to BCNU and NSC-135758

Treatment: ip				L1210/0 (ip; 10^5 cells)			L1210/BCNU (ip; 10^5 cells)		
Agent	Dosage (mg/kg/dose)	Schedule	Drug toxicity control* survivors/total	% ILS (excluding "cures")	Survivors/ total	Approximate No. of cells alive at end of treatment†	% ILS (excluding "cures")	Survivors/ total	Approximate No. of cells alive at end of treatment†
BCNU	40	Day 2 only	10/10	+105	6/10	3 (0.5)	+56	0/10	2.1×10^3
	30		10/10	+100	3/10	6 (1)	+37	0/10	2.2×10^4
NSC-135758	11.6	Day 2 only	7/10	+55	1/10	Toxic	+112	0/10	Toxic
	9.7		10/10	+55	0/10	1.6×10^3	+62	0/10	1.0×10^3
	6.5		10/10	+83	2/10	51 (2)	+100	0/10	10
	4.0	qd, 2-10 days	7/10	+77	0/10	Toxic	+137	0/10	Toxic
	2.6		10/10	+105	0/10	1.9×10^5	+100	0/10	2.2×10^6
	2.2		10/10	+122	0/10	2.5×10^4	+87	0/10	1.0×10^7
	1.5		10/10	+77	0/10	6.2×10^5	+62	0/10	2.2×10^8
	1.0		10/10	+44	0/10	3.9×10^8	+37	0/10	5.0×10^8

*Non-tumor-bearing (normal) mice.
†Based on median day of death in dying mice. Figures in parentheses are based on % survivors.

TABLE 6.—Comparative activity of NSC-135758 and CCNU against ic implanted L1210 leukemia*

Treatment: ip			Drug toxicity¶ control survivors/total	% ILS (excluding "cures")	60th-day survivors/total	Approximate No. of cells alive at end of treatment
Agent	Dosage (mg/kg/dose)	Schedule†				
CCNU	50	Day 1 only	10/10	0	9/10	0.1‡
NSC-135758	8	Day 1 only	10/10	+5	0/10	1.9×10^{4}§
	4		10/10	0	0/10	3.6×10^{4}§
	2		10/10	0	0/10	3.6×10^{4}§
	2	qd, 1–9 days	9/10	+30	0/10	2.3×10^{7}§
	1		10/10	+10	0/10	3.0×10^{8}§
	0.5		10/10	+10	0/10	3.0×10^{8}§
CCNU	50	Day 2 only	10/10	+110	9/10	0.1‡
NSC-135758	11.6	Day 2 only	7/10	+30	0/10	Toxic
	9.7		10/10	+30	0/10	1.9×10^{3}§
	6.5		10/10	+20	0/10	1.0×10^{4}§

*Each mouse was implanted ic with 10^{4} L1210 cells.
†Day 1 only treatment and Day 2 only treatment from 2 separate experiments.
‡Based on 60th-day survivors ("cures").
§Based on median lifespan of dying animals only.
¶Non-tumor-bearing (normal) mice.

TABLE 7.—Therapeutic activity of CPA, BCNU, L-PAM, and NSC-135758 vs L1210/0, L1210/CPA, and L1210/BCNU*

	Drug			
Leukemia line	CPA	BCNU	L-PAM	NSC-135758
L1210/0	+	+	+	+
L1210/CPA	–	+	+	+
L1210/BCNU	+	–	+	+

*+ = therapeutically active; – = therapeutically inactive.

BCNU is much less schedule-dependent for optimal therapeutic activity against L1210/0 (6).

Probably the most interesting and important data obtained from these studies are those that indicate that L1210/CPA and L1210/BCNU are as sensitive to NSC-135758 as the parent L1210/0 from which they were derived (tables 4 and 5).

The therapeutic activity of CPA, BCNU, L-PAM, and NSC-135758 against L1210/0, L1210/CPA, and L1210/BCNU is shown in table 7. Pertinent to these observations is the fact that L1210/CPA is as sensitive as L1210/0 to BCNU and L-PAM (table 2), and L1210/BCNU is as sensitive as L1210/0 to CPA and L-PAM (6). These observations clearly indicate likely differences in the cytotoxic biologic activity of NSC-135758 when compared with BCNU (and other nitrosoureas) and with CPA.

Schmid and Hutchison (11) reported that L1210/CPA was moderately cross-resistant to NSC-135758, and Tarnowski et al (12) observed that eight lines of L1210/CPA treated with single optimal doses of NSC-135758 showed an ILS of 33%–113%; with L1210/0 they reported a 113% ILS. However, the L1210/CPA lines of Tarnowski et al were not as markedly resistant to CPA as was the one we used in our studies.

Whether NSC-135758 has a different biologic mechanism of action from that of L-PAM is not apparent at this time. It is clearly different in biologic activity from the nitrosoureas, since it is active against L1210/BCNU and inactive against ic implanted L1210/0. Its biologic activity is different from that of CPA, since it is active against L1210/CPA. We have isolated a subline of L1210 which is less sensitive than L1210/0 to treatment with L-PAM.[5] This subline (L1210/L-PAM) retains marked sensitivity to NSC-135758.[5] Studies on the activity of NSC-135758 and other alkylating agents against L1210/L-PAM are under way and will be the subject of a later report. We are also studying CPA plus NSC-135758 in combination against L1210/0. John-

[5]When BDF_1 mice are implanted ip with 10^{6} L1210/0 or 10^{6} L1210/L-PAM cells and treated with equitoxic (≤ LD10) doses of L-PAM, 1000–10,000 times more L1210/L-PAM than L1210/0 cells survive drug treatment. Unpublished data from the Southern Research Institute.

son et al (13) and we (14) have reported marked therapeutic synergism of CPA plus L-PAM against L1210/0, leukemia P388 (14), and Ridgway osteogenic sarcoma (15).

Since L1210/L-PAM is sensitive to NSC-135758, different biologic mechanisms of action between L-PAM and NSC-135758 are indicated, and a new class of biologically active alkylating agents may have been demonstrated. These studies are of great potential importance, especially since NSC-135758 has been reported to have promising clinical activity against several histologically different types of advanced tumors in a number of patients, most of whom had had extensive prior chemotherapy (16).

LIST OF COMPOUNDS

BCNU: NSC-409962; CAS reg. No. 154-93-8; urea, 1,3-bis(2-chloroethyl)-1-nitroso-

CCNU: NSC-79037; CAS reg. No. 13010-47-4; urea, 1-(2-chloroethyl)-3-cyclohexyl-1-nitroso-

Cyclophosphamide: NSC-26271; CAS reg. No. 6055-19-2; 2*H*-1,3,2-oxazaphosphorine, 2-[bis(2-chloroethyl)amino]tetrahydro-, 2-oxide, monohydrate

Melphalan: NSC-8806; CAS reg. No. 3223-07-2; alanine, 3-[*p*-[bis(2-chloroethyl)amino]phenyl]-, monohydrochloride, L-; L-PAM

Methyl-CCNU: NSC-95441; CAS reg. No. 33073-59-5; urea, 1-(2-chloroethyl)-3-(4-methylcyclohexyl)-1-nitroso-

NSC-135758: CAS reg. No. 41109-80-2; 2,5-piperazinedione, 3,6-bis(5-chloro-2-piperidyl)-, dihydrochloride

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Comparison of 5-Fluorouracil and Ftorafur. I. Quantitative and Qualitative Differences in Toxicity to Mice[1,2,3,4]

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SUMMARY

5-Fluorouracil (5-FU) was compared to ftorafur, a fraudulent nucleoside analog which acts as a depot form of 5-FU, with respect to influence of dosage level and schedule of administration on toxicity in mice. When the drugs were administered daily and the duration of treatment was varied from a single dose to 32 daily treatments, the toxicity of 5-FU proved to be somewhat more than cumulative. On the other hand, the toxicity of ftorafur on daily treatment was less than cumulative. As a single treatment ftorafur was about half as toxic as 5-FU on an mg/kg basis. When the drugs were administered daily for 12 days the LD50 of ftorafur was about sevenfold that of 5-FU. When the drugs were administered as two treatments and the interval between the two treatments was varied from 0 to 14 days, it was found that 5-FU was considerably more toxic at an interval of 2–7 days than when two doses were administered simultaneously. Appreciable recovery of mice from the initial dose of 5-FU did not occur until sometime between 7 and 10 days. Host recovery from ftorafur toxicity was apparent by 2 days. The dose-mortality curves for both drugs were steep. The decreased toxicity with repeated treatment with ftorafur relative to 5-FU was not due to a decrease in the metabolism of ftorafur to 5-FU, which was much more extensive in mice compared to previously reported pharmacokinetic studies in other species. At equitoxic doses on a daily × 5 schedule, 5-FU and ftorafur had similar myelosuppressive activity. Ftorafur was, however, clearly less suppressive to both humoral and cell-mediated immunity than was 5-FU.

[Cancer Treat Rep 60:1335–1345, 1976]

Ftorafur is an analog of the antineoplastic antimetabolite 5-fluorouracil (5-FU). Ftorafur was synthesized by Hiller and co-workers (1) in 1966 and proved to be active in a spectrum of experimental tumors (2–7). Ftorafur has been clinically evaluated against solid tumors in the Soviet Union (8–11), Japan (12–18), and the US (19,20). Ftorafur, like 5-FU, was effective against breast (8–10,14,21) and gastrointestinal tumors (9,15,17,20).

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[3]5-Fluorouracil: NSC-19893; CAS reg. No. 51-21-8. Supplied by Hoffmann-La Roche, Inc, Nutley, NJ.

Ftorafur: NSC-148958; CAS reg. No. 17902-23-7; uracil, 5-fluoro-1-(tetrahydro-2-furyl)-. Provided by the Institute of Organic Synthesis, Academy of Sciences of the Latvian SSR, Riga, USSR.

[4]Dedicated to the memory of the late Professor S. A. Hiller, Institute of Organic Synthesis, Academy of Sciences of the Latvian SSR, Riga, USSR, in whose laboratory ftorafur was synthesized and who provided so much support and encouragement to collaborative US-USSR investigations.

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Although ftorafur was originally designed to act directly as an inhibitor of thymidylate synthetase or thymidine kinase (1), evidence suggests that it acts as a depot form of 5-FU. Ftorafur is a very weak pyrimidine antagonist in vitro as measured by the extent of inhibition of formate incorporation into DNA thymidine (22). Inhibition of deoxyuridine incorporation into DNA of cultured cells was evident only after long periods of exposure (23), at which time hydrolysis of the pseudonucleoside bond of ftorafur to yield 5-FU was demonstrated (22,23).

Ftorafur is metabolized to 5-FU in a number of species (24–31), including man (24,31). Homogenates of several tissues, particularly liver, promote the conversion of ftorafur to 5-FU (25). The plasma half-life of 5-FU is short, approximately 10 minutes in experimental animals (32–34) and man (35–37). Ftorafur has a plasma half-life of several hours in the rat (29), rabbit (24), dog (31), and man (24,31). 5-FU derived from ftorafur is present in plasma at significant levels for extended periods.

One of the major problems limiting the chemotherapeutic management of neoplastic disease is the toxicity of anticancer agents to vital tissues of the host. Most effective anticancer agents are toxic to the hematopoietic system and one of the dose-limiting toxicities is often myelosuppression. This is the case with 5-FU (38). A number of recent clinical trials (8,10,14,15,19–21) have indicated that ftorafur is effective against solid tumors and clearly less myelosuppressive than the parent compound. However, in one report (19) two patients receiving eight daily infusions of ftorafur (80 mg/kg/day) died, one patient from severe thrombocytopenia and one from severe leukopenia and septicemia. Daily infusions of 60 mg/kg did not cause significant myelosuppression. Novitskii (39,40) compared the myelosuppressive effects of therapeutic dose levels of ftorafur and 5-FU in rats and found that the effects of ftorafur were not as severe as those of 5-FU. Hršak and Pavičič (41) compared the effects of the two drugs on hematopoietic stem cells and peripheral blood components in mice and found that ftorafur and 5-FU had similar effects.

Another undesirable toxic effect of antineoplastic agents is immunosuppression. There is ample evidence suggesting a crucial role for the host immune system in eradicating residual tumor cells after reduction of tumor by cytotoxic agents or other treatment modalities (42,43). The suppressive effects of antineoplastic agents on immune response could interfere with the complete eradication of tumor (44,45). Thus, it is important to find analogs or new agents which are less immunosuppressive than the drugs which are presently available. 5-FU has been shown to be immunosuppressive in experimental animals and man (46–48).

A series of studies were performed in experimental animals to compare the toxic and therapeutic properties of ftorafur and 5-FU. In this paper we describe studies of the influence of dosage level and schedule of administration on the lethal toxicity of ftorafur and 5-FU in mice. Experiments were designed to assess cumulative toxicity from multiple-dose treatment and to compare the rate of host recovery from single-dose toxicity of the drugs. The observed decreased toxicity of ftorafur relative to 5-FU on repeated treatment led us to evaluate the pharmacokinetics of ftorafur in mice after a single dose or daily treatment. The myelosuppressive and immunosuppressive activities of equitoxic doses of 5-FU and ftorafur were evaluated. In the following paper (7) 5-FU and ftorafur were compared with respect to therapeutic activity, schedule dependency, and resistance in experimental tumors.

MATERIALS AND METHODS

Animals.—Six- to 8-week-old (20–25 g) male B6D2F$_1$ (C57BL/6 female × DBA/2 male), C3H, and C57BL/6 mice were used in all experiments. Groups of five to eight mice were housed in plastic cages and allowed pelleted feed and water ad libitum. The mice were obtained from the production colonies of the Drug Research and Development Program (DR&DP), Division of Cancer Treatment (DCT), National Cancer Institute (NCI).

Drugs.—5-FU was dissolved in sterile 0.85% NaCl to an appropriate concentration so that the desired dose was administered in a volume of 0.01 ml/g of body weight. For high doses (> 300 mg/kg) of 5-FU which were not soluble in the standard injection volume, 0.02 ml/g of body weight of drug solution was administered. Ftorafur was dissolved in sterile 0.85% NaCl solution by dropwise addition of 5 N NaOH to pH 9.5. The desired doses were administered in a volume of 0.01 ml/g of body weight. Drugs were administered sc unless otherwise noted. 2-(^{14}C)-ftorafur (9.3 millicuries/mmol) was obtained from the DR&DP, DCT, NCI.

Toxicity studies.—Toxic dose levels of 5-FU and ftorafur on a given schedule were determined from dose-response curves. Seven to nine logarithmically spaced doses were used to generate each dose-response curve. Doses were closely spaced; each succeeding dose was 1.25-fold greater than the preceding dose. A group of six B6D2F$_1$ mice was treated at each dose level. The mice were observed for 45 days from initiation of treatment. LD50 dose levels and slopes of dose-response curves were determined by Kärber's method (49). In order to compare the

cumulative toxicity of 5-FU and ftorafur, the LD50 levels were determined for a single injection and for daily treatment for 2, 4, 6, 8, 12, 16, and 32 days. In a second type of experiment the time required for mice to recover from an initial dose of 5-FU or ftorafur was compared by determination of the LD50 dose levels of two injections spaced at progressively longer intervals: 0–5, 7, 10, and 14 days.

Pharmacokinetic study.—To determine whether there was reduced metabolism of ftorafur to 5-FU after repeated ftorafur administration, two groups of B6D2F$_1$ mice were given chromatographically pure (as determined by thin-layer chromatography on the system described by Cohen [29]) 2-(^{14}C)-ftorafur (100 microcuries/100 mg/kg iv). Animals in one group had received 100 mg/kg of unlabeled ftorafur ip daily for 4 days prior to injection of labeled drug. Animals in the other group had not been treated prior to receiving labeled ftorafur. Three mice from each group were exsanguinated via the brachial artery and vein under ether anesthesia at 1, 2, 4, 8, 16, 24, and 48 hours. Blood was collected in heparinized tubes and plasma was separated by centrifugation. Plasma levels of total radioactivity, ftorafur, and 5-FU were determined by thin-layer chromatography on silica gel plates developed with a solvent system consisting of ethyl acetate/methanol/ammonium hydroxide (75:25:1, vol/vol/vol) and liquid scintillation counting as described by Cohen (29).

Peripheral leukocyte and thrombocyte counts.—B6D2F$_1$ mice were bled from the retro-orbital sinus into heparinized capillary tubes at intervals after drug administration. A different group of animals was bled at each time point. Leukocytes and thrombocytes were counted with an electronic particle counter (Model ZBi, Coulter Electronics, Hialeah, Fla). Differential counts of lymphocytes and granulocytes were made on smears of whole blood stained with Giemsa stain.

Assay of humoral immune response.—Sheep erythrocytes (SRBC) (Flow Laboratories, Rockville, Md) were washed three times with 0.85% NaCl prior to iv injection of 0.25 ml of a 30% (vol/vol) suspension in C3H mice. Groups of five mice were bled from the retro-orbital sinus at weekly intervals and sera were collected. The micro-hemagglutination assay described by Stavitsky (50) was used to evaluate the sera. The serum dilution that produced 50% agglutination of SRBC compared to the serum from non-immunized controls was considered as the end point and the titer reported is the reciprocal of this dilution.

Assay of cell-mediated immune response.—Cell-mediated immunity was evaluated by an allograft rejection assay. A subline of L1210 leukemia resistant to 5-FU, and cross-resistant to ftorafur (7), was implanted ip at three inoculum levels in allogeneic C57BL/6 mice. Suppression of the cell-mediated immune response is reflected in a greater proportion of mice (bearing a given tumor inoculum) failing to reject the tumor and dying with progressive tumor growth.

RESULTS

Cumulative Toxicity of 5-FU and Ftorafur

On daily treatment the toxicity of 5-FU was more cumulative than that of ftorafur. The LD50 dose level of 5-FU decreased more sharply with repeated injections than that of ftorafur (fig 1A). This is more evident when the data are plotted as total-dose LD50 versus the number of daily injections (fig 1B). For 5-FU there was somewhat more than additive toxicity for four, six, eight, 12, and 16 daily treatments since the total-dose LD50 was less than the LD50 for a single injection. For ftorafur, on the other hand, the total dose LD50 increased progressively with the number of daily injections, and on no schedule was greater than additive toxicity observed.

The ratio of toxic dose levels of ftorafur to 5-FU was dependent on the duration of daily treatment (table 1). As a single sc treatment, 5-FU was about twice as potent as ftorafur with regard to lethal toxicity. On repeated treatment, due to differences

TABLE 1.—LD10 and LD90 dose levels of 5-FU and ftorafur in B6D2F$_1$ male mice on daily treatment of varying duration

Schedule: qd ×—	5-FU LD10 (mg/kg/injection)	5-FU LD90 (mg/kg/injection)	5-FU LD90/LD10	Ftorafur LD10 (mg/kg/injection)	Ftorafur LD90 (mg/kg/injection)	Ftorafur LD90/LD10	Ratio of LD50 of ftorafur/LD50 of 5-FU
1	305	458	1.50	632	847	1.34	1.91
2	134	250	1.87	382	475	1.24	2.20
4	64.1	94.5	1.47	289	373	1.29	4.10
6	41.9	62.0	1.48	197	239	1.21	4.28
8	30.0	38.8	1.29	191	230	1.20	5.54
12	20.4	27.7	1.35	151	197	1.30	7.20
16	19.6	21.4	1.09	102	162	1.59	6.67
32	18.8	20.6	1.09	87.2	124	1.42	5.30

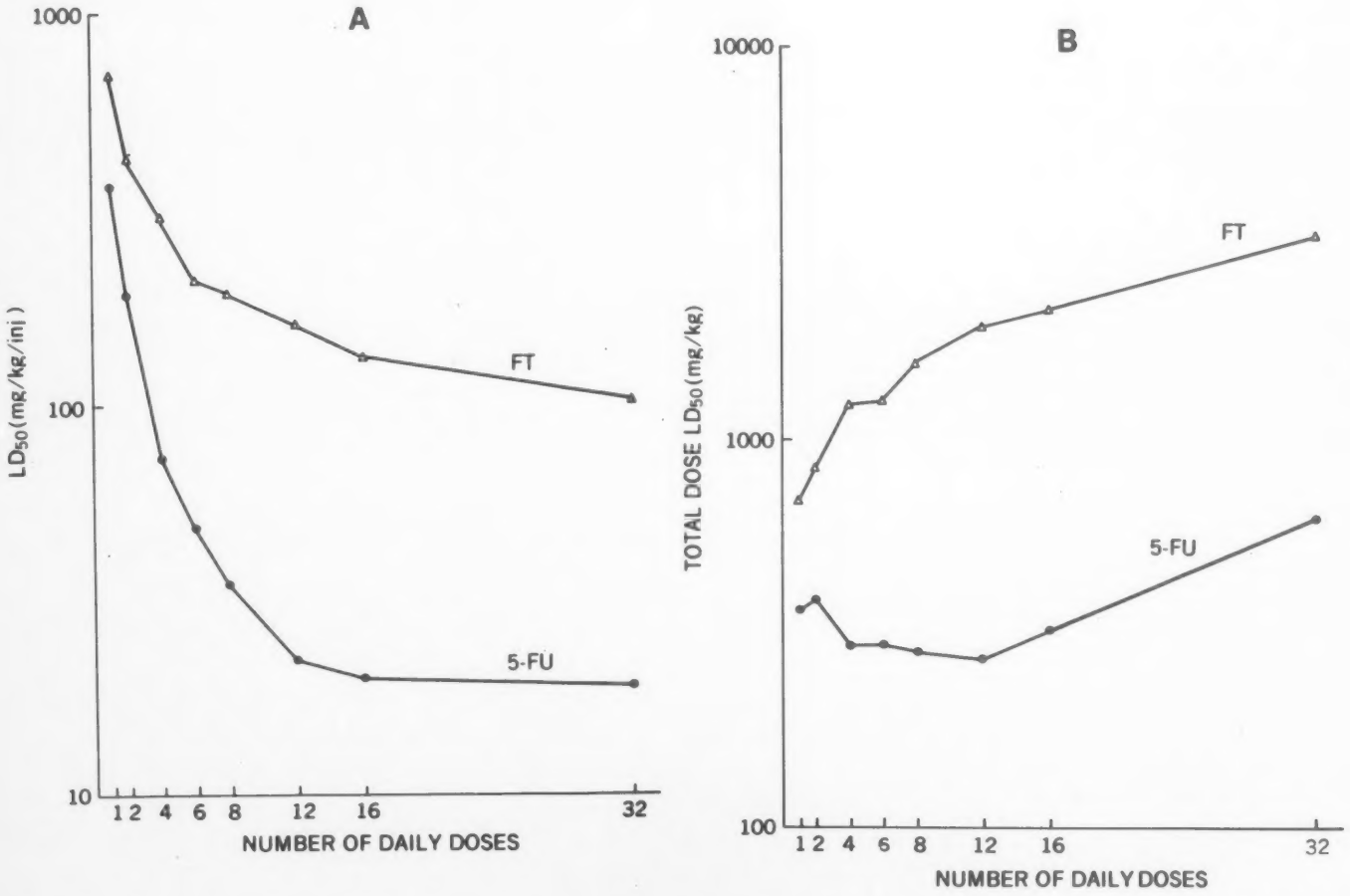

FIGURE 1.—Comparison of the cumulative toxicity of 5-FU and ftorafur on daily treatment of varying duration in B6D2F$_1$ male mice. *Panel A:* LD50 dose level as a function of number of daily treatments. *Panel B:* Total-dose LD50 as a function of number of daily treatments, calculated by multiplying the LD50 dose level on a given schedule by the number of injections.

in cumulative toxicity, the difference in potency of the two analogs increased to about sevenfold for 12 or 16 daily injections. The ratio fell to 5.3 for 32 daily injections.

The slopes of the dose-mortality curves for 5-FU and ftorafur on the various daily treatment schedules were very steep. The LD10 and LD90 dose levels are shown in table 1. On most schedules the LD90/LD10 ratio was < 1.5. In general, the slopes of the mortality curves for ftorafur were somewhat steeper than those for 5-FU. However, the slopes for 5-FU administered daily for 16 or 32 days were exceedingly steep (LD90/LD10 = 1.09).

The central nervous system toxicity of ftorafur which has been noted in man (20) was evidenced as lethargy and ataxia in mice immediately after individual doses of ≥ 250 mg/kg. However, this toxicity was transient and did not appear to be related to the lethal effects of the drug. Toxic dose levels of 5-FU and ftorafur on the various schedules killed the mice at approximately the same time. The mean time to death from initiation of treatment of mice which received lethal doses as one, two, four, six, eight, or 12 daily treatments was between 8 and 13 days. Toxic doses of both drugs caused a progressive weight loss.

Host Recovery From Toxicity

The interval between two doses of 5-FU had a dramatic effect on the toxicity of this antimetabolite. Two doses spaced at 1–7 days were considerably

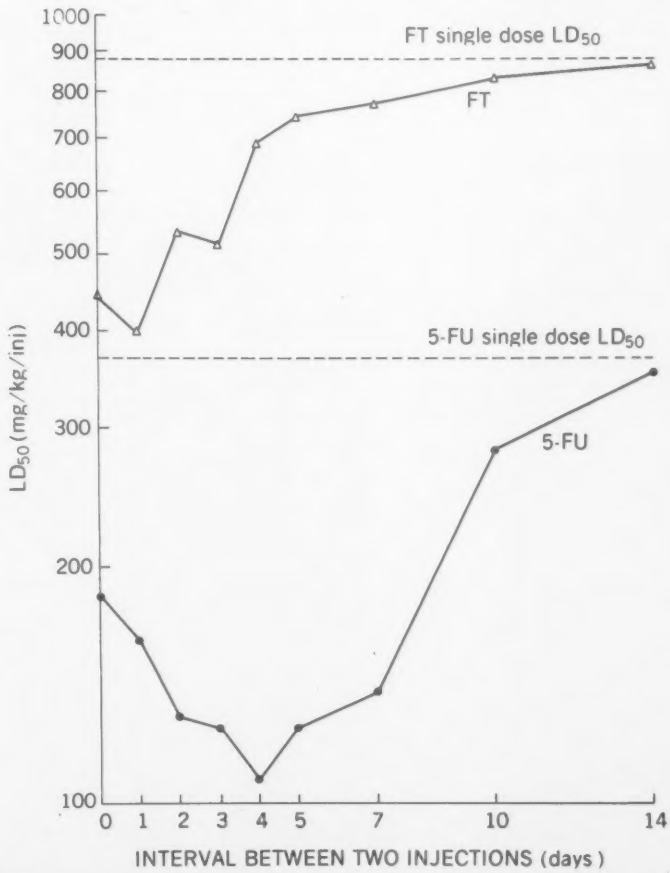

FIGURE 2.—Recovery of B6D2F$_1$ male mice from toxicity of 5-FU and ftorafur. Two treatments were administered at the intervals indicated. The horizontal-dashed lines represent LD50s for a single dose which is equivalent to twice the LD50 for two treatments given simultaneously (0-day interval). As the interval is increased to infinity the curves should approach but not surpass the single-dose LD50 since the first treatment at this dose would have killed 50% of the treated animals and the second treatment would kill 50% of the remaining animals.

more toxic than when the two doses were administered simultaneously (an interval of 0 days, equivalent to one half of the single-dose LD50) (fig 2). The greatest toxicity for 5-FU was observed when two doses were administered with a 4-day interval. For 5-FU, recovery did not occur until sometime between 7 and 10 days. The recovery curve for ftorafur was distinctly different (fig 2). There was only a slight increase in toxicity relative to a single dose (0-day interval) when the interval was 1 day. The animals recovered from ftorafur toxicity by 2 days as the LD50 surpassed the LD50 for a 0-day interval and continued to increase as the interval was lengthened.

The ratio of toxic dose levels of ftorafur to 5-FU reflects the differences in host recovery (table 2). A single dose of 5-FU was 2.4-fold more toxic than ftorafur on an mg/kg basis. The ratio increased to 6.4-fold when two doses were given with a 4-day interval. The ratio returned to 2.4 when the interval was 14 days, suggesting that the two doses of the drugs were acting independently at this interval.

As in the previous experiment the slopes of the dose-mortality curves were steep (table 2). Again, the slopes of the mortality curves for ftorafur were, on most schedules, somewhat steeper than those for 5-FU.

Metabolism of Ftorafur With Single- and Multiple-Dose Therapy

One possible explanation for differences in cumulative toxicity and host recovery between 5-FU and ftorafur could be that ftorafur serves as a self-limiting depot form of 5-FU, ie, on repeated treatment there is less breakdown of ftorafur to 5-FU. To test this possibility the pharmacokinetics of 2-(^{14}C)-ftorafur was compared in previously untreated mice

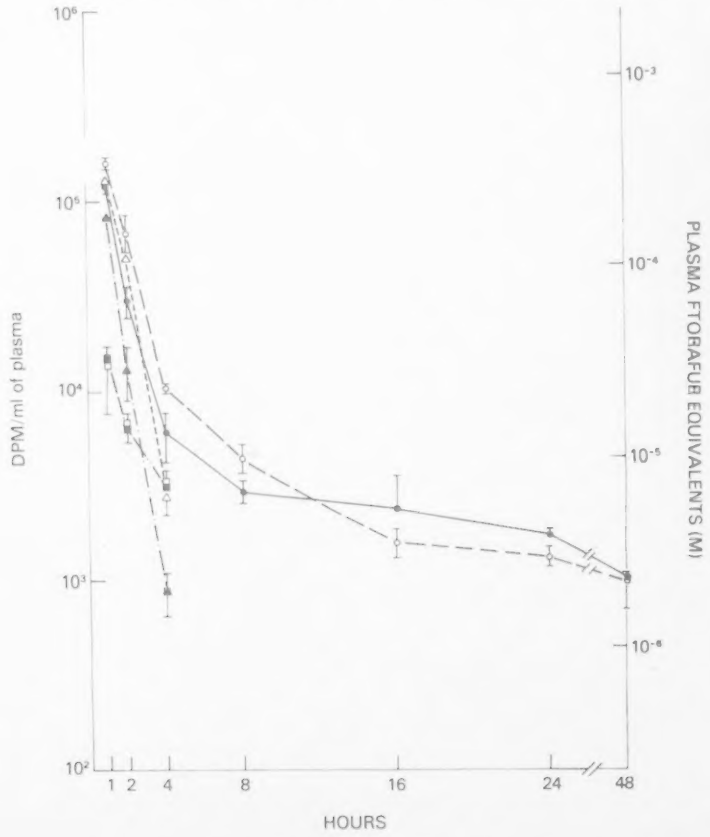

FIGURE 3.—Plasma disappearance curves of total radioactivity, ftorafur, and 5-FU after iv administration of 2-(^{14}C)-ftorafur to groups of mice which were either untreated or previously treated with ftorafur for 4 days. ●——● = Total radioactivity; ▲—·—·▲ = ftorafur; and ■—·—··■ = 5-FU in the previously untreated group. ○------○ = Total radioactivity; △------△ = ftorafur; and □------□ = 5-FU in the group previously treated with ftorafur.

TABLE 2.—LD10 and LD90 dose levels of 5-FU and ftorafur in B6D2F$_1$ male mice on 2 treatments spaced at varying intervals

Schedule	5-FU LD10 (mg/kg/injection)	5-FU LD90 (mg/kg/injection)	5-FU LD90/LD10	Ftorafur LD10 (mg/kg/injection)	Ftorafur LD90 (mg/kg/injection)	Ftorafur LD90/LD10	Ratio of LD50 of ftorafur/LD50 of 5-FU
q0d × 2	160	229	1.43	404	527	1.31	2.39
qd × 2	130	230	1.77	365	467	1.28	2.44
q2d × 2	119	150	1.26	450	649	1.44	4.12
q3d × 2	107	154	1.44	412	621	1.51	4.09
q4d × 2	97.0	125	1.29	600	736	1.23	6.44
q5d × 2	105	151	1.44	695	909	1.31	5.93
q7d × 2	125	202	1.61	710	927	1.30	5.56
q10d × 2	245	368	1.50	750	951	1.27	2.95
q14d × 2	308	470	1.52	750	979	1.30	2.44

and in mice which had been treated with unlabeled ftorafur for 4 days. The plasma disappearance curves for total plasma radioactivity, ftorafur, and 5-FU for these two groups of animals are shown in figure 3. There were no apparent differences between the treated and previously untreated mice. The apparent volume of distribution of ftorafur was 555 ml/kg in both groups. The initial half-lives of total plasma radioactivity and of ftorafur were 43 and 38 minutes respectively in the treated group compared to 45 and 34 minutes respectively in the previously untreated group. Plasma levels of 5-FU at 1, 2, and 4 hours were identical in the two groups. Because of the small plasma volume and the low level of radioactivity the identity of plasma radioactivity could not be determined chromatographically at time points beyond 4 hours.

Effect of 5-FU and Ftorafur on Peripheral Leukocyte and Thrombocyte Counts

LD10 dose levels of both 5-FU (50 mg/kg) and ftorafur (200 mg/kg) administered daily for 5 days produced severe leukopenia in B6D2F$_1$ mice (table 3). A nadir of 20% of control for 5-FU and 15% of control for ftorafur was observed 7 days after initiation of treatment. Differential leukocyte counts revealed that both drugs caused a profound depletion of granulocytes. Lower doses of 5-FU and ftorafur had a comparable moderate and transient effect on the total leukocyte count but still produced severe granulocytopenia 7 days after initiating treatment.

At the LD10 dose levels both drugs caused a decrease in the thrombocytes by about 90% on Day 10 (table 3). At lower dose levels 5-FU depleted the thrombocytes to a greater extent than did ftorafur.

Effect of 5-FU and Ftorafur on Humoral Immunity to SRBC

5-FU and ftorafur were administered to groups of C3H mice at equitoxic doses daily for 5 days beginning the day after challenge with SRBC. The hemagglutination titer of serum was determined 7, 14, and 21 days after this challenge. Mice which received no drug had a median hemagglutination titer of 128 at each of these time points (table 4). Serum

TABLE 3.—Effects of daily treatment with 5-FU and ftorafur on peripheral leukocytes and thrombocytes of B6D2F$_1$ mice*

Drug	Dose (mg/kg/injection, sc, Days 0–4)	Days after initiation of treatment: 4 Wbc† (% granulocytes)	4 Platelets†	7 Wbc† (% granulocytes)	7 Platelets†	10 Wbc† (% granulocytes)	10 Platelets†	14 Wbc† (% granulocytes)	14 Platelets†
5-FU	50	47 (25)	28	20 (2)	21	24 (3)	12	78 (10)	160
	30	87 (9)	86	55 (1)	22	100 (5)	41	99 (27)	249
	18	53 (8)	73	77 (2)	45	117 (19)	117	113 (31)	153
Ftorafur	200	32 (16)	51	15 (3)	17	33 (2)	8	119 (11)	175
	120	46 (8)	98	66 (6)	70	115 (16)	83	93 (30)	109
	72	67 (10)	107	92 (8)	87	142 (24)	63	88 (17)	83

*Average values of 8 untreated control mice: leukocyte count = 9430 ± 680/μl^3, 16% ± 1% granulocytes; platelet count = 398,000 ± 28,000/μl^3.

†Percent of control leukocyte and thrombocyte counts. Each value represents the average of 5 mice.

TABLE 4.—Effects of daily treatment with 5-FU and ftorafur on humoral immune response of C3H mice to SRBC*

Drug	Dose (mg/kg/injection, sc, Days 1–5)	Median hemagglutination titer (range of individual titers): days after SRBC challenge*		
		7	14	21
Normal controls (no SRBC)		Negative		
Untreated controls (SRBC only)		128 (32–128)	128 (32–128)	128 (64–128)
5-FU	50	Negative	Negative	Negative
	30	Negative (negative–8)	2 (2–8)	4 (4–8)
	18	16 (16–64)	32 (16–64)	16 (16–64)
Ftorafur	200	4 (negative–64)	16 (4–128)	32 (4–64)
	120	64 (32–64)	64 (64–128)	64 (64–128)
	72	128 (64–128)	128 (32–128)	64 (64–128)

*SRBC administered iv on Day 0. Hemagglutination titers determined from individual sera of 5 mice at each time point.

from mice which had been given an LD10 dose level of 5-FU (50 mg/kg/injection) did not agglutinate SRBC. Two lower doses of 5-FU (0.6 LD10 and 0.36 LD10) also strongly suppressed the humoral immune response to SRBC at all time points. Comparable doses of ftorafur had a lesser effect on humoral immunity. An LD10 dose level (200 mg/kg/injection) was less suppressive than a 30-mg/kg/injection of 5-FU. The median hemagglutination titers of serum from mice treated with the two lower doses of ftorafur were within one dilution of serum of the untreated controls indicating little or no suppression of humoral immunity.

Effect of 5-FU and Ftorafur on Cell-Mediated Immunity

To compare the effects of the two drugs on cell-mediated immunity, the influence of drug administration on the rejection of an allogeneic tumor was determined. A subline of L1210 leukemia completely resistant to 5-FU and completely cross-resistant to ftorafur (7) was chosen to eliminate any direct cytotoxic effects of the drugs on the tumor cells. 5-FU and ftorafur were administered daily for 5 days beginning 2 days prior to tumor inoculation. In untreated allogeneic C57BL/6 mice the tumor was rejected in all animals challenged with 8.5×10^6 or 1×10^6 viable tumor cells (table 5). One of eight mice challenged at 1×10^5 cells did not reject the tumor. Cell-mediated immunity was strongly suppressed in mice treated with an LD10 of 5-FU, since all of the mice at each tumor challenge level died from progressive tumor growth. The lower doses of 5-FU were also quite immunosuppressive since eight of eight mice at 30 mg/kg and seven of eight mice at 18 mg/kg failed to reject the highest tumor challenge. 5-FU at 30 mg/kg also prevented tumor rejection by a majority of mice at the lower tumor challenge levels.

Ftorafur was much less immunosuppressive in this assay. An LD10 dose level (200 mg/kg) allowed tumor growth in, at the most, three of eight mice which received the various challenge levels of the allogeneic tumor. The two lower doses of ftorafur had little or no effect on cell-mediated immunity. In nontumored mice tested in parallel to this experiment, no more than one of eight mice treated with any of the doses of 5-FU or ftorafur died from drug toxicity.

DISCUSSION

These studies show that there are considerable differences in the toxicity of ftorafur and 5-FU in mice. While 5-FU was more potent with regard to lethal toxicity than ftorafur on all of the schedules tested, there were some schedules of administration that maximized the difference in potency between the two compounds. When the congeners were administered daily for 12 days or twice with a 4-day interval between doses, 5-FU was six to seven times as potent as ftorafur on a weight basis. The smallest difference in lethal dose levels was obtained when the agents were administered as a single dose. This twofold difference on a weight basis approaches equivalent toxicity on a molar basis, taking into account the increased molecular weight of ftorafur (200.2) relative to 5-FU (130.1).

The schedule-dependent variation of relative toxicity of 5-FU and ftorafur was due to distinct differences in the toxicity of repeated doses. 5-FU admin-

TABLE 5.—Effects of daily treatment with 5-FU and ftorafur on cell-mediated immune response as measured by rejection of an allogeneic tumor

Drug	Dose (mg/kg/injection, sc, Days −2–+2)	Mortality (No. of mice that died/total No.) according to tumor challenge (L1210/5-FU cells/mouse, ip)*		
		8.5×10^6	1×10^6	1×10^5
Untreated controls		0/8	0/8	1/8
Cyclophosphamide	180 (Day −1)	8/8	8/8	8/8
5-FU	50	8/8	8/8	8/8
	30	8/8	5/8	5/8
	18	7/8	1/8	0/8
Ftorafur	200	3/8	2/8	3/8
	120	1/8	1/8	2/8
	72	1/8	1/8	0/8

*L1210/5-FU-resistant cells implanted ip on Day 0 in allogeneic C57BL/6 mice. 8 of 8 syngeneic $B6D2F_1$ mice died from progressive tumor growth when inoculated with 10^6 or 10^5 L1210/5-FU cells ip.

istered daily had greater than cumulative toxicity while ftorafur toxicity was not cumulative. Daily 5-FU treatment was slightly less than cumulative only when the drug was administered for 32 days. This is possibly because there is a threshold dose of approximately 18 mg/kg of 5-FU which could be administered daily for an indefinite period without toxicity (*see* table 1). The greater than cumulative toxicity of 5-FU is probably a reflection of the pharmacokinetics of the agent. Although 5-FU has a very short plasma half-life, significant levels of the drug and its active anabolites are retained in tissues for long periods (32). It might be expected that a depot form of 5-FU, such as ftorafur (22,23), would show the same pattern of cumulative toxicity or, perhaps, be even more cumulative. One possible explanation for this discrepancy would be that the lethal toxicity of ftorafur could be due to an independent pharmacologic action of the drug itself which overshadows any toxicity due to the 5-FU released as a metabolite. However, the observation that equitoxic dose levels of ftorafur and 5-FU on a 5-day treatment schedule resulted in severe leukopenia and thrombocytopenia (table 3) suggests that, in mice, myelosuppression is an important toxic manifestation of both drugs.

Van Putten et al (51) compared the effects of ftorafur and 5-FU on hematopoietic and leukemic stem cells. They showed that, as a single dose, ftorafur was about twofold to threefold less potent than 5-FU. However, with multiple-dose treatment, they found that ftorafur was much less potent than 5-FU. The differences in cumulative toxicity described herein support these observations.

The experiment in which the analogs were administered with an interval varying from 0 to 14 days between two doses (fig 2) shows that mice recover from sublethal toxicity of ftorafur much more rapidly than from that of 5-FU. The latter agent was most toxic when two doses were given at an interval of 4 days. This is possibly due to rebound proliferation of vital stem cells after destruction of the actively proliferating marrow and/or gastrointestinal epithelium stem cell pool by the initial dose of 5-FU. Eaves and Bruce (52) showed that hematopoietic stem cells were highly sensitive to 5-FU after they had been stimulated to proliferate by treatment with endotoxin, cyclophosphamide, or radiation. There was only a slight increase in ftorafur toxicity when two doses were given at a 1-day interval and progressively less toxicity at longer intervals. The differences in host recovery from the two analogs could be explained by the observations of Van Putten et al (51) that, while ftorafur was only two to three times less potent than 5-FU in killing hematopoietic stem cells, there was a sevenfold difference in potency in the killing of rapidly proliferating hematopoietic stem cells. Thus, ftorafur would not have the increased toxicity during rebound proliferation of stem cells which was seen with 5-FU.

The marked differences in cumulative toxicity and host recovery raise the question of whether ftorafur acts solely as a depot form of 5-FU. It is quite evident that ftorafur has no innate cytotoxic activity and must be metabolically activated (23,30). One of the active metabolites is clearly 5-FU (and subsequent active anabolites of 5-FU) (26,29,31). However, Smolyanskaya and Tugarinov (30), using microbiologic assays, found another cytotoxic component in the blood and tissues of ftorafur-treated mice which was distinct from 5-FU.

A possible explanation, originally proposed by Van Putten et al (51), which could account for the

differences observed in the toxicity of ftorafur and 5-FU, would be that an initial dose of ftorafur might bring about adaptation of the host so that, on subsequent treatment, there would be less 5-FU released. We evaluated this possibility by measuring the plasma disappearance curves of ftorafur and 5-FU after administration of labeled ftorafur to previously untreated and ftorafur-treated mice. Host adaptation was not observed; the plasma levels of 5-FU were similar in the two groups of mice.

The pharmacokinetic study revealed that conversion of ftorafur to 5-FU is much greater in mice than in rats (29), rabbits (24), dogs (31), and man (24,31). Except in mice, ftorafur itself is the major component in plasma and has a half-life of several hours; 5-FU accounts for only a small percentage of plasma radioactivity. In mice, on the other hand, ftorafur had a plasma half-life of about 0.5 hour and 5-FU was the predominant plasma metabolite at 4 hours after administration of ftorafur.

The comparison of the effects of multiple injections of 5-FU and ftorafur reported in this paper indicates that 5-FU is more suppressive to both the humoral and cell-mediated immune systems in mice than is ftorafur at equitoxic doses (tables 4 and 5). Previous studies on the effects of 5-FU on immune response in mice have yielded conflicting results (46,53,54). Immunosuppression by 5-FU is clearly dependent on the dosage level and schedule of administration used (46,53).

With ftorafur as well, immunosuppressive activity was directly related to dosage level. However, an LD10 dose level of ftorafur was clearly less suppressive to both humoral and cell-mediated immunity than was 5-FU at doses at or below the LD10 level. Assuming that the immune response in cancer patients plays an important role in eradicating residual tumor cells after drug-induced tumor regression, as has been suggested by experimental models (43,44), reduced immunosuppressive activity of an analog with proven clinical activity, such as ftorafur, should be a distinct advantage.

The finding of significant myelosuppression by ftorafur in mice (table 3), which was similar to that of 5-FU at equitoxic doses, confirms the report of Hršak and Pavičič (41). These authors observed moderate leukopenia and thrombocytopenia following administration of 5-FU and ftorafur for 6 days at 23 and 133 mg/kg respectively. These doses are close to the 0.6 LD10 doses used in the present study.

In clinical trials ftorafur was considerably less myelosuppressive than conventional 5-FU therapy (8,10,14,15,19–21). These results are similar to what was observed in rats but are in conflict with the studies in mice. A possible explanation for this species difference might be the more extensive conversion of ftorafur to 5-FU in mice (fig 3) compared to rats (29) or man (24,31).

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Comparison of 5-Fluorouracil and Ftorafur. II. Therapeutic Response and Development of Resistance in Murine Tumors[1,2,3,4]

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SUMMARY

The therapeutic activity of ftorafur was compared to that of 5-fluorouracil (5-FU) in a number of tumor systems. The drugs were active against ip L1210 leukemia when administered ip, sc, or orally. Administration every fourth day × 3 proved to be the most effective treatment schedule for both drugs, although significant activity was seen on all treatment schedules tested. Both congeners had activity against sc implanted L1210 leukemia as well as a limited effect on the ic implanted tumor. 5-FU produced greater increases in lifespan of mice bearing L1210 leukemia than did ftorafur. 5-FU was also more effective against ip B16 melanoma and ip Gardner 6C3HED lymphosarcoma. Ftorafur was ineffective in the treatment of mice bearing ip P388 leukemia, a tumor which is quite sensitive to 5-FU. At approximately equimolar doses both drugs produced a persistent inhibition of 2′-deoxyuridine incorporation into DNA of L1210 cells in vivo. Ftorafur produced a greater inhibition of uridine incorporation into RNA than did 5-FU, which may account for the lower therapeutic activity of ftorafur. In combination chemotherapy of L1210 leukemia 5-FU plus ftorafur was no more effective than 5-FU alone, neither of the congeners was synergistic with either adriamycin or actinomycin D, and in combination with methotrexate therapeutic synergism was observed with 5-FU but not with ftorafur. After eight transplant generations of exposure to ftorafur, a subline of L1210 leukemia became totally resistant to ftorafur and simultaneously cross-resistant to 5-FU. Doses of ftorafur and 5-FU which were optimally effective in mice bearing the parental L1210 line were lethal to mice implanted with the ftorafur-resistant subline. When treatment of the resistant subline was discontinued after nine transplant generations of exposure to ftorafur, sensitivity to 5-FU returned after three transplant generations without ftorafur. The subline retained its resistance to ftorafur until eight transplant generations after cessation of ftorafur treatment. Another subline of L1210 leukemia exposed to 5-FU for 20 transplant generations proved to be completely resistant to 5-FU and cross-resistant to ftorafur. The mutual cross-resistance between ftorafur and 5-FU supports the contention that ftorafur acts primarily as a depot form of 5-FU.

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[3]5-Fluorouracil: NSC-19893; CAS reg. No. 51-21-8. Supplied by Hoffmann-La Roche, Inc, Nutley, NJ.

Ftorafur: NSC-148958; CAS reg. No. 17902-23-7; uracil, 5-fluoro-1-(tetrahydro-2-furyl)-. Provided by the Institute of Organic Synthesis, Academy of Sciences of the Latvian SSR, Riga, USSR.

[4]Dedicated to the memory of the late Professor S. A. Hiller, Institute of Organic Synthesis, Academy of Sciences of the Latvian SSR, Riga, USSR, in whose laboratory ftorafur was synthesized and who provided so much support and encouragement to collaborative US-USSR investigations.

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Ftorafur is an analog of 5-fluorouracil (5-FU) synthesized by Hiller and co-workers (1) which serves as a slow-release depot form of 5-FU (2–7). Ftorafur has activity comparable to 5-FU in human breast and gastrointestinal carcinomas with less myelosuppression than is associated with conventional 5-FU therapy (8–12). The activity and toxicity of ftorafur in man (12) are similar to results obtained with continuous iv infusions of 5-FU (13,14).

Studies in experimental tumor systems (15–18) have shown that, in most cases, ftorafur is active against tumors which are sensitive to 5-FU. However, a rigorous comparison of the therapeutic efficacy of these two analogs in a number of tumor systems has not, heretofore, been undertaken.

The schedule of administration of a number of antineoplastic agents has been shown to have a considerable influence on therapeutic efficacy (19). The activity of 5-FU in human cancer has not been shown to be markedly dependent on the schedule of treatment (20). However, ftorafur might have schedule-dependency characteristics quite different from 5-FU since schedule dependency is related to the pharmacokinetics of an agent as much as to the mechanism of action (19). Examples would be the dissimilarity in schedule dependency of cytosine arabinoside in comparison to its depot forms, cyclocytidine (21) and cytosine arabinoside-5′-adamantoate (22). Studies described in the preceding paper (7) of the influence of schedule on toxicity of 5-FU and ftorafur in mice suggested that, on certain treatment schedules, the toxicity of ftorafur is reduced relative to 5-FU. In this report, the activity of 5-FU and ftorafur against ip implanted L1210 leukemia is compared on a variety of treatment schedules.

Since ftorafur is being incorporated into a number of combination regimens in the clinic on the basis of its decreased myelosuppression relative to 5-FU (12), we compared the effects of the two agents in combination with each other and with a number of other antitumor agents in L1210 leukemia.

The failure of a chemotherapeutic agent or combination of agents to maintain remission, or to induce regression of a tumor which had recurred after initially effective therapy, is a critical problem in the chemotherapy of human cancer. The failure of previously effective therapy can be due to a number of factors including selection of biochemically resistant cells, sequestration of tumor in sites where effective drug levels are not attained, changes in kinetics of the tumor cell population, and adaptation of tumor cells by enzyme induction.

Biochemical resistance, both acquired after chemotherapy and occurring naturally in the absence of prior treatment, has been reported in a number of cases of human cancer (23–26). The selection of mutant cells resistant to antineoplastic agents has been studied extensively in microorganisms and animal tumors (27,28). Resistance to 5-FU has been related to deletion of enzymes required for anabolism to 5-fluoro-2′-deoxyuridine-5′-monophosphate (FdUMP) (29–31) and to changes in the binding affinity of thymidylate synthetase for FdUMP (32). Cross-resistance between 5-FU and the closely related analogs, 5-fluoro-2′-deoxyuridine (FUdR) and 5-fluorouridine, has been demonstrated (30).

Since most evidence suggests that ftorafur acts as a slow-release depot form of 5-FU (4,7,33,34), cross-resistance between ftorafur and 5-FU might be expected. To establish this point we developed sublines of L1210 leukemia resistant to either ftorafur or 5-FU and evaluated the sublines for cross-resistance to the other analog.

MATERIALS AND METHODS

Animals.—Male B6D2F$_1$ (C57BL/6 female × DBA/2 male) and CD2F$_1$ (BALB/c female × DBA/2 male) mice and female C3H and DBA/2 mice, obtained from the production colonies of the Drug Research and Development Program (DR&DP), Division of Cancer Treatment (DCT), National Cancer Institute (NCI), were used in these studies. The mice were 6–8 weeks old (20–25 g) at the time of tumor inoculation.

Drugs.—5-FU was dissolved in 0.85% NaCl for injection into mice. Ftorafur was suspended in distilled water and dissolved by adjusting to pH 9.5 by dropwise addition of 5 N NaOH. Methotrexate, actinomycin D, and adriamycin[11] were obtained from the DR&DP, DCT, NCI. These agents were dissolved for injection in 2% sodium bicarbonate solution, 10% ethanol solution, and distilled water respectively. Drug solutions were prepared to an appropriate concentration so that the desired dose was administered in a volume of 0.01 ml/g of body weight. The route of drug administration was ip unless specifically noted in the tables and figures.

Tumors.—L1210, P388, and P1534 leukemias, B16 melanoma, Lewis lung carcinoma, and Gardner 6C3HED lymphosarcoma were maintained by continuous passage in syngeneic mice and implanted into test mice according to protocols of the DR&DP, DCT, NCI (35). In chemotherapy experiments L1210 leukemia was implanted ip (10^5 ascitic cells), sc (10^6 ascitic cells), or ic (0.05 ml of leukemic blood, approximately 5×10^5 cells) in B6D2F$_1$ mice. P388 leukemia and Gardner 6C3HED lymphosarcoma were inocu-

[11]Methotrexate: NSC-740; CAS reg. No. 59-05-2; glutamic acid, *N*-[*p*-[[(2,4-diamino-6-pteridinyl)methyl]methylamino]benzoyl]-.
Actinomycin D: NSC-3053.
Adriamycin: NSC-123127; CAS reg. No. 23214-92-8.

lated ip (10^6 ascitic cells) in $B6D2F_1$ and C3H mice respectively. P1534 leukemia was implanted ip in $B6D2F_1$ mice by inoculating 0.2 ml of a 1:5 (wt/vol) brei of spleens from tumor-bearing donor mice. A single-cell suspension of Lewis lung carcinoma (2×10^6 cells in 0.1 ml) was implanted im in the thigh of $B6D2F_1$ mice. A crude 1:5 (wt/vol) brei of B16 melanoma was implanted ip (0.5 ml) or sc (0.2 ml) in $B6D2F_1$ mice. A subline of L1210 leukemia resistant to ftorafur was developed in $B6D2F_1$ hybrid mice. On successive transplant generations 10^5 cells were implanted ip into groups of six mice and 100 mg/kg of ftorafur was administered ip daily for 9 days. Mice were killed on Day 11 and tumor cells were collected for the subsequent transplant generation. The daily dose of ftorafur for maintenance of the resistant subline was reduced to 65 mg/kg at the eighth and subsequent transplant generations due to the observed enhancement of toxicity in mice bearing the resistant tumor. When complete resistance to ftorafur had appeared at the eighth transplant generation an additional group of $B6D2F_1$ mice was implanted ip with 10^5 cells of the resistant subline. This group did not receive daily ftorafur treatment and was used to show whether the resistance to the drug was stable. This subline was serially transplanted along with the subline that was continually exposed to ftorafur. A subline of L1210 leukemia resistant to 5-FU was developed in $CD2F_1$ mice by exposure to 5-FU on successive transplant generations. 5-FU at 10 mg/kg/day was administered daily for 9 days after ip implantation of 10^6 cells. Mice were killed when ascites was evident and cells were collected for inoculation of the subsequent transplant generation.

Therapeutic studies.—The drugs were administered via the routes and on the treatment schedules indicated in the tables and figures over a wide range of logarithmically spaced dosage levels. Each treated group consisted of eight mice and untreated control groups were made up of 16–40 mice. Antitumor activity was assessed by the median increase in lifespan (ILS) relative to untreated controls. The relative effectiveness of 5-FU and ftorafur was based on the results obtained with the optimal dose which produced the maximum median ILS. 5-FU and ftorafur were evaluated in combination chemotherapy regimens against L1210 leukemia implanted ip. The analogs were tested in combination with each other and with methotrexate, actinomycin D, and adriamycin. Combination regimens were administered on an intermittent treatment schedule (Days 1, 5, and 9) at various dosage ratios based on anticipated optimal dose levels: (*a*) 0.25 optimal dose of drug A + optimal dose of drug B; (*b*) 0.5 optimal dose of drug A ± 0.5 optimal dose of drug B; and (*c*) optimal dose of drug A + 0.25 optimal dose of drug B (36). A regimen was considered to be therapeutically synergistic if the combination resulted in an ILS which was greater than that achieved by the optimal dose of the most effective agent used individually.

Sensitivity of resistant sublines to 5-FU and ftorafur.—On every second transplant generation throughout the development of the ftorafur-resistant subline, groups of eight $B6D2F_1$ mice were implanted ip with 10^5 cells of either the parental L1210 tumor or the ftorafur-resistant subline. The mice were then treated ip with ftorafur and 5-FU on Days 1, 5, and 9 at several dose levels to determine the extent of resistance to these drugs. This schedule proved to be optimal for these drugs in the treatment of the sensitive L1210 line. The lifespan of the treated mice was determined as a measure of antitumor activity. The effects of 5-FU and ftorafur were compared in the sensitive L1210 leukemia and in the subline resistant to 5-FU in its 20th transplant generation. The sensitive line (10^5 cells) and the 5-FU-resistant subline (10^6 cells) were implanted ip in groups of eight $B6D2F_1$ mice. The higher inoculum level of the resistant subline was used to achieve equivalent median survival times (MST) of untreated controls bearing the resistant and sensitive sublines. 5-FU and ftorafur were evaluated on three treatment schedules: (*a*) Day 1 only; (*b*) every fourth day, Days 1, 5, and 9; and (*c*) daily, Days 1–9.

Nucleic acid synthesis by L1210 leukemia in vivo.—$B6D2F_1$ mice were inoculated ip with 10^5 L1210 cells and were treated 5 days later with approximately equimolar doses of the drugs (180 mg/kg of 5-FU or 300 mg/kg of ftorafur). At various time points after drug administration the mice were injected ip with 20 microcuries of 6-(^{3}H)-deoxyuridine (specific activity 28 curies/mmol, New England Nuclear, Boston, Mass) and 1 microcurie of 2-(^{14}C)-uridine (specific activity 6.5 millicuries/mmol, New England Nuclear). After a pulse period of 30 minutes the mice were killed by cervical dislocation and ascitic tumor cells were aspirated in 0.85% NaCl. The cells were collected by centrifugation at $600 \times g$ for 10 minutes and resuspended in 2.0 ml of saline. The cells were disrupted and macromolecules precipitated by homogenization after addition of 0.2 ml of 70% cold perchloric acid (PCA). The PCA-insoluble fraction was collected by centrifugation, washed twice in 2 ml of cold 5% PCA, and resuspended in 0.8 ml of 1.5% PCA. Nucleic acids were extracted by heating this suspension to 90°C for 20 minutes. The mixture was centrifuged after addition of 0.1 ml of cold 70% PCA. The pellet was extracted again by repeating the heating procedure in 1.5% PCA and the supernatants, containing solubilized nucleic

TABLE 1.—Influence of schedule and route of administration on the activity of 5-FU and ftorafur against ip implanted L1210 leukemia

Schedule of administration	Route of administration	5-FU					Ftorafur				
		Dose (mg/kg/injection)		Survival time at optimal dose (days)			Dose (mg/kg/injection)		Survival time at optimal dose (days)		
		Range	Optimal	Range	Median	%ILS	Range	Optimal	Range	Median	%ILS
Daily, Days 1–9	ip	9–65	23	10–23	13.5	59	14–108	108	10–17	12.0	41
Day 1 only	ip	39–833	300	9–16	15.0	76	65–1300	833	8–19	12.0	41
q3h, Day 1 only	ip	3–39	39	7–22	13.5	59	5–65	65	7–15	12.0	41
q3h, Days 1,5,9	ip	3–23	14	10–22	18.5	118	5–39	39	2–16	14.0	65
Days 1,5,9	ip	23–500	65	13–23	19.0	123	39–833	500	9–16	14.0	65
Days 1,5,9	sc	23–500	65	15–19	16.0	89	39–833	500	15–18	17.0	100
Days 1,5,9	oral	23–500	108	13–18	16.0	89	39–833	500,833	10–15	14.0	65
Untreated controls				7–14	8.5				7–14	8.5	

acids, were combined. Aliquots of the PCA-soluble nucleic acid fraction were used for determination of DNA by the diphenylamine assay (37) and RNA by the orcinol assay (38). Further 0.5-ml aliquots were added to 15 ml of Aquasol (New England Nuclear) and incorporated radioactivity was determined by double-label counting in a Beckman LS-250 liquid scintillation counter. The incorporation of 6-(^{3}H)-deoxyuridine into DNA and 2-(^{14}C)-uridine into RNA was calculated as dpm of ^{3}H/μg DNA and dpm of ^{14}C/μg of RNA respectively. Control animals were injected with saline instead of drug solutions and were evaluated throughout the course of the experiment. The specific activities of DNA and RNA from eight control animals were 3589 ± 533 dpm/μg of DNA and 89 ± 6 dpm/μg of RNA. The results are expressed as the percent of control nucleic acid biosynthesis and each point represents the average of duplicate determinations of three mice.

RESULTS

Therapeutic activity against L1210 leukemia.—5-FU and ftorafur were subjected to detailed comparative evaluation against L1210 leukemia. The results of a study designed to evaluate the influence of schedule and route of administration on therapeutic activity against ip L1210 leukemia are shown in table 1. Both drugs had significant activity on all treatment schedules studied. When administered ip, 5-FU was most effective on an intermittent treatment schedule (Days 1, 5, and 9); the ILS was 123% at the optimal dose. A similar ILS (118%) was obtained when 5-FU was administered every 3 hours for eight injections on Days 1, 5, and 9. Similarly, on ip administration of ftorafur, maximum prolongation of survival time (65% ILS) was obtained when the drug was administered either every 3 hours or as a single dose on Days 1, 5, and 9. Both drugs retained activity when administered sc or orally on Days 1, 5, and 9. 5-FU was somewhat less effective by these routes compared to ip administration. Ftorafur was most effective when administered sc (100% ILS). 5-FU was more effective than ftorafur on all treatment schedules with the exception of sc treatment on Days 1, 5, and 9. 5-FU and ftorafur were also evaluated against sc and ic implanted L1210 leukemia (table 2). Both drugs were active when administered either sc or ip on Days 1, 5, and 9 against the sc tumor. 5-FU produced greater ILSs than ftorafur. Both drugs retained some degree of antileukemic activity against the ic implanted tumor indicating that the congeners are capable of passing the blood-brain barrier. 5-FU was again more effective than ftorafur.

Effects on incorporation of pyrimidine nucleoside precursors in DNA and RNA of L1210 cells in vivo.—The time course of inhibition of the incorporation of deoxyuridine into DNA and uridine into RNA after a single dose of 5-FU or ftorafur to mice bearing advanced ip L1210 leukemia is shown in figure 1. The incorporation of deoxyuridine into DNA of L1210 cells was rapidly and completely inhibited by both drugs. Essentially complete inhibition of deoxyuridine incorporation was observed at all time points from 1 hour to 4 days following drug administration. The inhibition of uridine incorporation into the RNA of the L1210 cells was both slower in onset and more transitory compared to effects on deoxyuridine incorporation. 5-FU inhibited uridine incorporation by about 70% for 8 hours after which time the rate of incorporation of this RNA precursor gradually returned to control levels. The inhibition of uridine incorporation by ftorafur was greater than by 5-FU. One hour after ftorafur administration there was a 70% inhibition which was similar to

TABLE 2.—Activity of 5-FU and ftorafur against L1210 leukemia implanted sc or ic

Site of tumor implantation	Treatment		5-FU			Ftorafur		
	Schedule (days)	Route	Dose (mg/kg/injection)		%ILS	Dose (mg/kg/injection)		%ILS
			Range	Optimal		Range	Optimal	
sc	1,5,9	ip	23–180	108	77	65–833	300	55
	1,5,9	sc	23–180	108	88	65–833	500	61
ic	1,5,9	ip	23–180	108	50	65–833	500	35
	1–9	ip	9–65	23	25	23–180	39	10

that of 5-FU. Thereafter, the rate of uridine incorporation continued to decline to 3% of control at 6 hours, followed by a gradual rise to control levels.

Therapeutic activity in a spectrum of murine tumors.—Comparative activity of 5-FU and ftorafur in six diverse tumor systems is shown in table 3. 5-FU had significant activity on two treatment schedules against ip implanted P388 leukemia; ftorafur was devoid of activity in this tumor system. B16 melanoma implanted ip responded to both congeners. However, as in L1210 leukemia, 5-FU was more effective than ftorafur. Neither drug had significant activity against im implanted Lewis lung carcinoma, sc implanted B16 melanoma, or P1534 leukemia. Gardner 6C3HED lymphosarcoma was very sensitive to 5-FU when the antimetabolite was administered ip; five of eight mice treated with 15 mg/kg on Days 1–5 survived 60 days with no evidence of tumor. When 5-FU was given sc there was only 41% ILS at the optimal dose. Ftorafur was inactive in this tumor system when administered ip and had only borderline activity (25% ILS) when administered sc.

Combination chemotherapy studies in L1210 leukemia.—5-FU and ftorafur were evaluated in combination with each other against ip L1210 leukemia (data not shown). The drugs were administered individually and in combination on Days 1, 5, and 9. 5-FU alone (60 mg/kg/injection) gave an 88% ILS. The optimal response to ftorafur was 39% ILS at 300 mg/kg. The combination resulted in a maximum response of 88% ILS, no better than that obtained with 5-FU alone. This response was obtained by combining one half of the optimal doses of the drugs used individually. Similar ILSs (78%) were obtained by combining an optimal dose of either of the two drugs with one fourth of the optimal dose of the other drug. Combinations of methotrexate with either 5-FU or ftorafur are shown in figure 2. Methotrexate alone (60 mg/kg, Days 1, 5, and 9) produced 111% ILS. The optimal response obtained with 5-FU was 78% ILS compared to 61% ILS for ftorafur. In this experiment the optimal dose of ftorafur shifted to 1200 mg/kg, the highest dose tested. Therapeutic synergism was obtained by combining optimal doses of methotrexate and 5-FU (167% ILS). On the other hand, the combination of methotrexate plus ftorafur was no better than methotrexate alone. The optimal response was obtained by combining half of the optimal doses of each agent. It is unlikely that a combination of full optimal doses of methotrexate and ftorafur would

TABLE 3.—Comparison of 5-FU and ftorafur against a spectrum of murine tumors

Tumor system		Treatment		5-FU			Ftorafur		
Tumor	Implantation site	Schedule (days)	Route	Dose (mg/kg/injection)		%ILS	Dose (mg/kg/injection)		%ILS
				Range	Optimal		Range	Optimal	
P388 leukemia	ip	1,5,9	ip	23–180	39	45	65–833	—	0
		1–9	ip	9–65	23	109	23–180	—	0
B16 melanoma	ip	1,5,9	ip	23–180	65	75	65–833	300	50
		1–9	ip	9–65	23	100	23–180	108	40
B16 melanoma	sc	1,5,9	ip	23–180	—	0	108–833	108	16
		1–9	ip	9–65	—	0	23–180	—	0
P1534 leukemia	ip	1,5,9	ip	23–180	23	11	108–833	—	0
		1–9	ip	9–65	9	24	23–180	—	0
Lewis lung carcinoma	im	5,9,13,17	ip	6–100	12	14	25–800	—	0
Gardner 6C3HED lymphosarcoma	ip	1–5	ip	8–60	15	> 275	38–300	—	0
		1–5	sc	8 –60	30	41	38–300	75	25

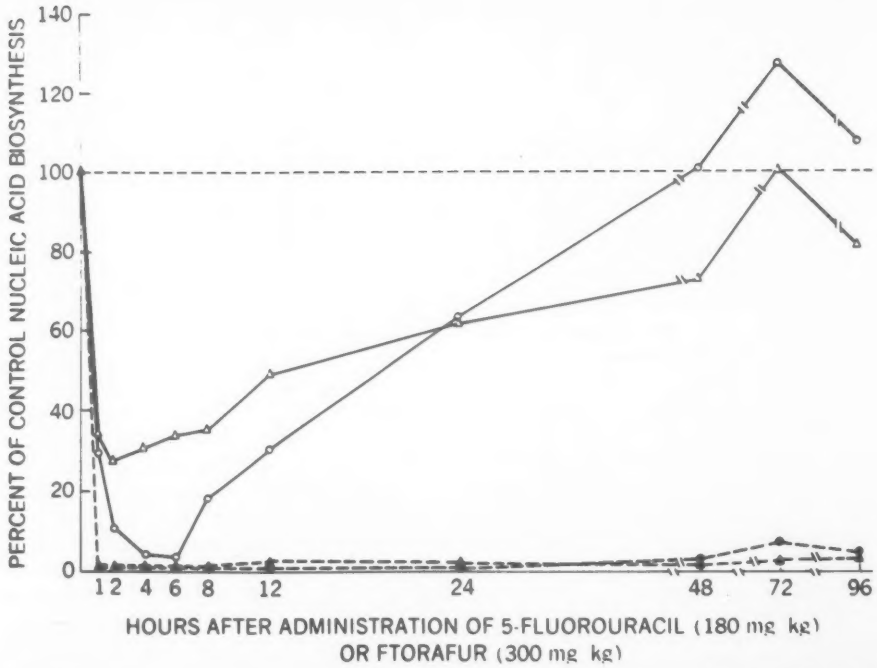
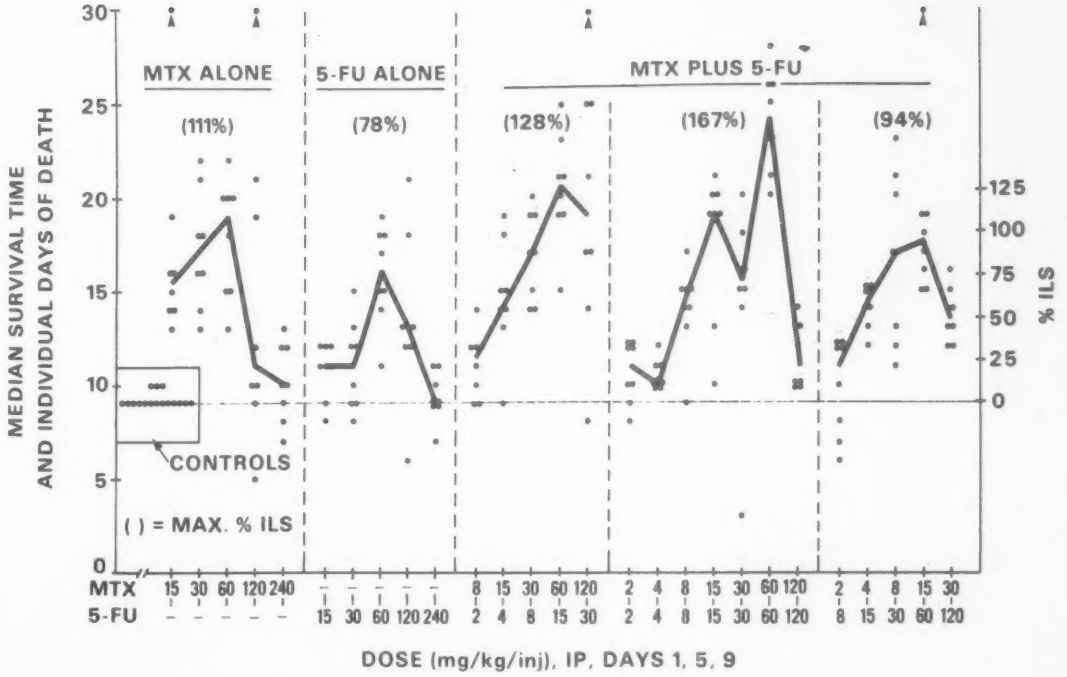


FIGURE 1.—Inhibition of incorporation of deoxyuridine and uridine into nucleic acids of ascitic L1210 leukemia cells in vivo after a single equimolar dose of 5-FU or ftorafur. Data expressed as percent of control specific activity of hot PCA-soluble nucleic acids after a 30-min in vivo pulse of labeled precursors. ▲—— ▲ = 5-FU, deoxyuridine incorporation; △——— △ = 5-FU, uridine incorporation; ●—— ● = ftorafur, deoxyuridine incorporation; ○——— ○ = ftorafur, uridine incorporation.

have given a greater response since 60 mg/kg of methotrexate plus 300 mg/kg of ftorafur showed some evidence of toxicity in this experiment. Combined therapy with adriamycin or actinomycin D plus either 5-FU or ftorafur showed no evidence of therapeutic synergism in L1210 leukemia (data not shown).

Development of a subline of L1210 leukemia resistant to ftorafur.—A subline of L1210 leukemia exposed to ftorafur (100 mg/kg/day) over four transplant generations had not become resistant to ftorafur. Furthermore, the subline retained its sensitivity to 5-FU. After six transplant generations of exposure to ftorafur, the L1210 subline appeared to have become simultaneously resistant to ftorafur and cross-resistant to 5-FU (fig 3A). Ftorafur increased the lifespan of mice bearing the resistant line by only 10% compared to 50% in mice bearing the parental L1210 line. A maximally tolerated dose of 5-FU produced 24% ILS in mice bearing the ftorafur-resistant line as opposed to 89% ILS in a parallel test against the parental line. Complete resistance to both 5-FU and ftorafur was evident after eight transplant generations of exposure to ftorafur (fig 3B). A peculiar phenomenon appeared and was evident in all subsequent transplant generations of the ftorafur-resistant line. Mice bearing the resistant line were acutely sensitive to toxic effects of both ftorafur and 5-FU. Doses which were not toxic and produced optimal therapeutic response in mice bearing the L1210 parental line (60 mg/kg of 5-FU, 400 mg/kg of ftorafur) killed mice bearing the resistant line 6–8 days after initiation of treatment. The mice showed no evidence of accelerated tumor growth; there were few ascites cells and no splenomegaly or hepatomegaly at the time of death. Routine culture of the tumor cells in thioglycollate broth showed no evidence of bacterial contamination. Mice treated with the lowest doses of the two drugs had extensive ascitic tumor growth at the time of death indicating that the subline was resistant to the drugs at maximally tolerated dose levels. A similar pattern of response to 5-FU and ftorafur was seen after ten (data not shown) and 12 (fig 4) transplant generations of exposure to ftorafur. After nine transplant generations the ftorafur-resistant line was split into two sublines, one of which was continually exposed to ftorafur. The other re-



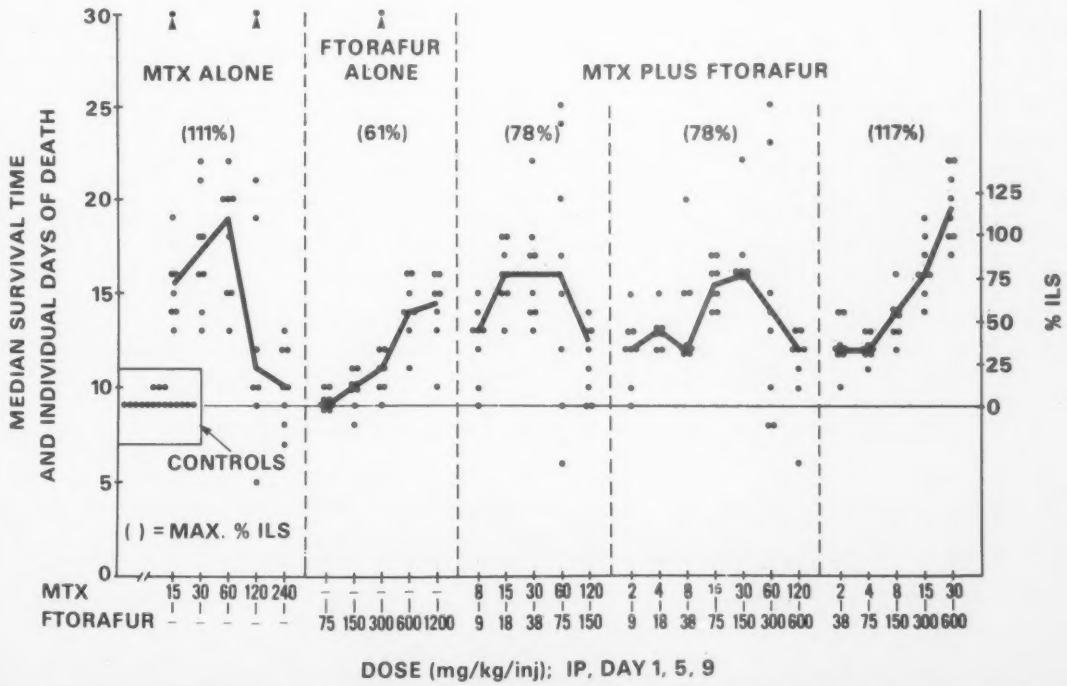


FIGURE 2.—Combination chemotherapy of ip L1210 leukemia with methotrexate plus either 5-FU or ftorafur. Data points represent survival time of individual mice and curves are drawn through MSTs of each treated group.

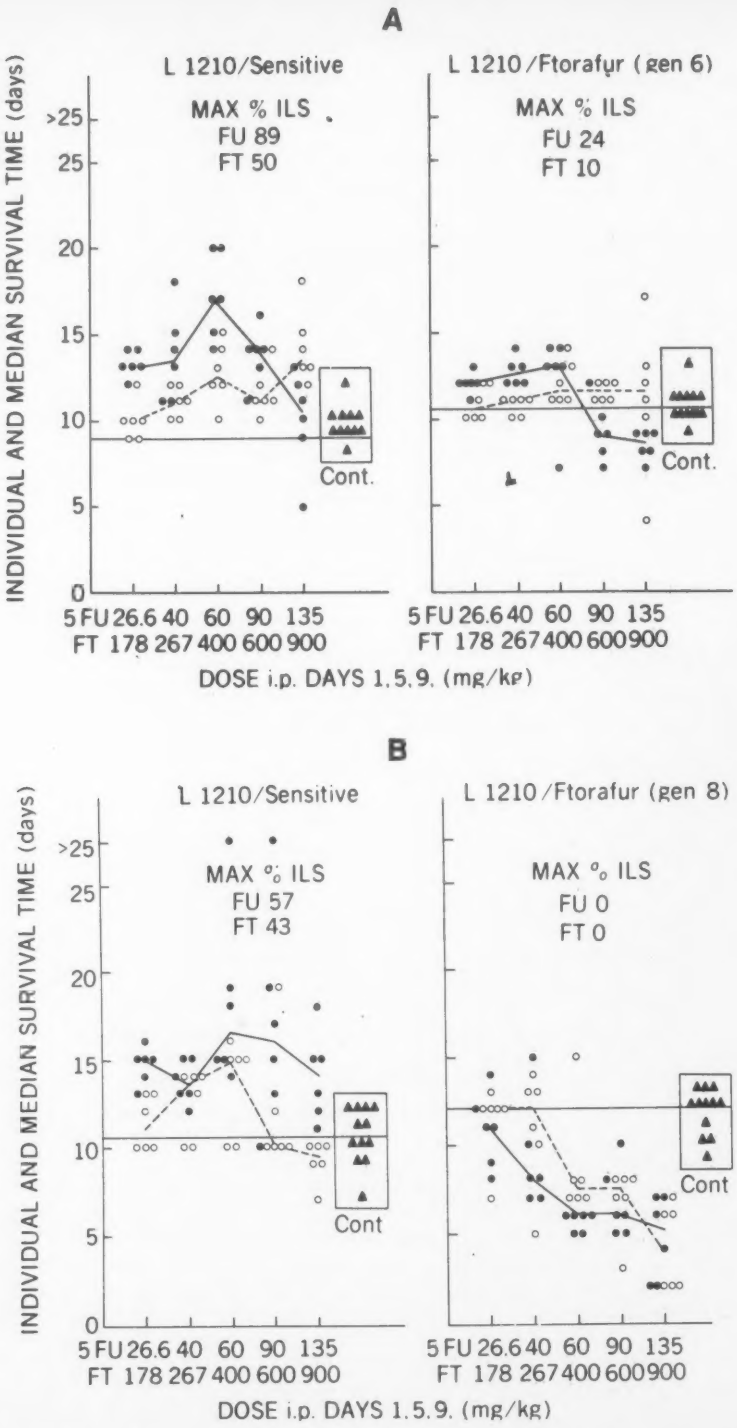


FIGURE 3.—Effects of 5-FU and ftorafur on the lifespan of mice bearing ip L1210 leukemia (L1210/sensitive) and a subline of L1210 leukemia exposed to ftorafur over 6 (A) or 8 (B) transplant generations. ▲ = untreated controls; ●——● = 5-FU; ○- - - - ○ = ftorafur. Each symbol represents an individual mouse. The solid and dashed lines are drawn through the MST at each dose level of 5-FU and ftorafur respectively.

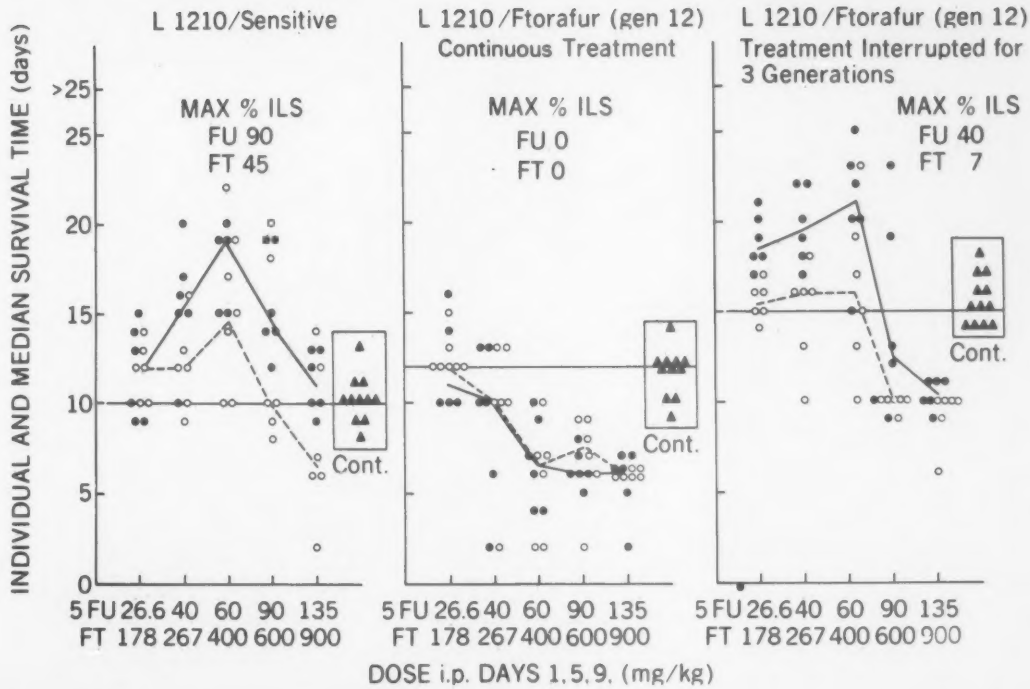


FIGURE 4.—Effects of 5-FU and ftorafur on the lifespan of mice bearing ip L1210 leukemia (L1210/sensitive), a subline of L1210 leukemia exposed to ftorafur over 12 transplant generations, and a subline exposed to ftorafur over 9 transplant generations and subsequently transplanted 3 generations in the absence of ftorafur.

ceived no further treatment to determine whether the resistance to ftorafur was stable. When ftorafur treatment had been discontinued for three transplant generations, sensitivity to 5-FU had partially returned, yet the subline retained resistance to ftorafur (fig 4). The enhancement of toxicity of the drugs was not evident in the subline which was not continually exposed to ftorafur. After 14 transplant generations (fig 5) the subline treated continuously with ftorafur remained totally resistant to both drugs. The subline which had been untreated for five transplant generations was still resistant to ftorafur but was quite sensitive to 5-FU; two of six mice treated with 5-FU at 40 and 60 mg/kg were long-term survivors. After 17 transplant generations of exposure to ftorafur (fig 6) the toxicity of ftorafur and 5-FU was even further enhanced since the mice treated with the lowest dose level of 5-FU (26.6 mg/kg) died prior to the untreated controls. In the subline which had not been exposed to ftorafur for eight transplant generations, it appeared that sensitivity to ftorafur was returning, although there was only 21% ILS at the maximally tolerated dose (fig 6). 5-FU produced a 62% ILS. The MST of untreated control mice bearing the resistant subline exposed to ftorafur on every transplant generation was, in general, about 2 days longer than untreated control mice receiving the same inoculum of parental L1210 cells (figs 3–6). However, after discontinuation of ftorafur treatment the untreated controls inoculated with 10^5 cells of the resistant subline tended to survive for somewhat longer periods of time: after three generations without ftorafur exposure the MST of untreated controls was 15 days (fig 4); after five generations, 16.5 days (fig 5); and after eight generations, 14.5 days (fig 6).

Cross-resistance to ftorafur of a subline of L1210 leukemia resistant to 5-FU.—A subline of L1210 leukemia exposed to 5-FU for 20 transplant generations was completely resistant to 5-FU on three treatment schedules (fig 7A). This subline proved to be cross-resistant to ftorafur (fig 7B). The 5-FU-resistant subline did not display the increased toxicity to 5-FU or ftorafur that was observed in treatment of mice bearing the ftorafur-resistant subline.

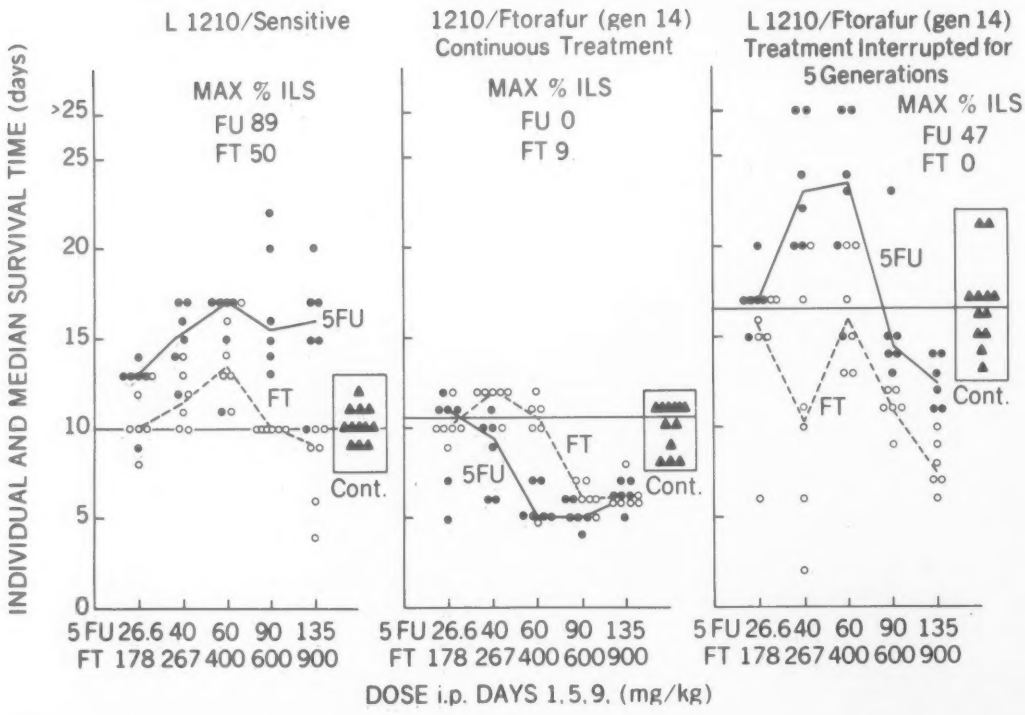


FIGURE 5.—Effects of 5-FU and ftorafur on the lifespan of mice bearing ip L1210 leukemia (L1210/sensitive), a subline of L1210 leukemia exposed to ftorafur over 14 transplant generations, and a subline exposed to ftorafur over 9 transplant generations and subsequently transplanted 5 generations in the absence of ftorafur.

DISCUSSION

5-FU and ftorafur had reproducible activity against L1210 leukemia. Although both analogs were most effective when administered on Days 1, 5, and 9, therapeutic activity was not markedly dependent on the schedule of administration (table 1). The drugs were also effective when administered as a single dose on Day 1 only, every 3 hours on Day 1 only or Days 1, 5, and 9, and daily on Days 1–9. These results correspond to clinical studies which have shown 5-FU to be effective when administered by prolonged infusion (13), as well as on daily treatment and intermittent high-dose schedules (20). Most clinical studies with ftorafur have been with daily treatment for 2–4 weeks (8,10,11,39) or treatment for 5–7 days repeated every 2–3 weeks (12,40). High-dose intermittent therapy or prolonged infusions with ftorafur have not been evaluated clinically.

Studies on the influence of schedule of administration on the relative toxicity of 5-FU and ftorafur to mice (7) indicated that there were some schedules which would maximize the difference in toxicity between the two compounds. For instance, when the drugs were administered daily × 12 or every 4 days × 2, ftorafur was about one sixth to one seventh as toxic as 5-FU on an mg/kg basis compared to only about a twofold difference in toxicity when the drugs were administered as a single treatment. This would suggest that ftorafur might have a therapeutic advantage over 5-FU on prolonged daily administration or intermittent (Days 1, 5, and 9) treatment. However, 5-FU was superior to ftorafur against L1210 on these schedules, as it was on single-dose treatment on Day 1 only. It would appear that the relative toxicity of the analogs against leukemic cells parallels the relative toxicity to the host. This conclusion is supported by the study of Van Putten et al (41) in which the effects of 5-FU and ftorafur on hematopoietic and leukemic stem cells were compared using the spleen colony assay. They observed that ftorafur was two to three times less potent than 5-FU in killing both stem cell populations when the drugs were administered as single treatments. When the drugs were administered in multi-

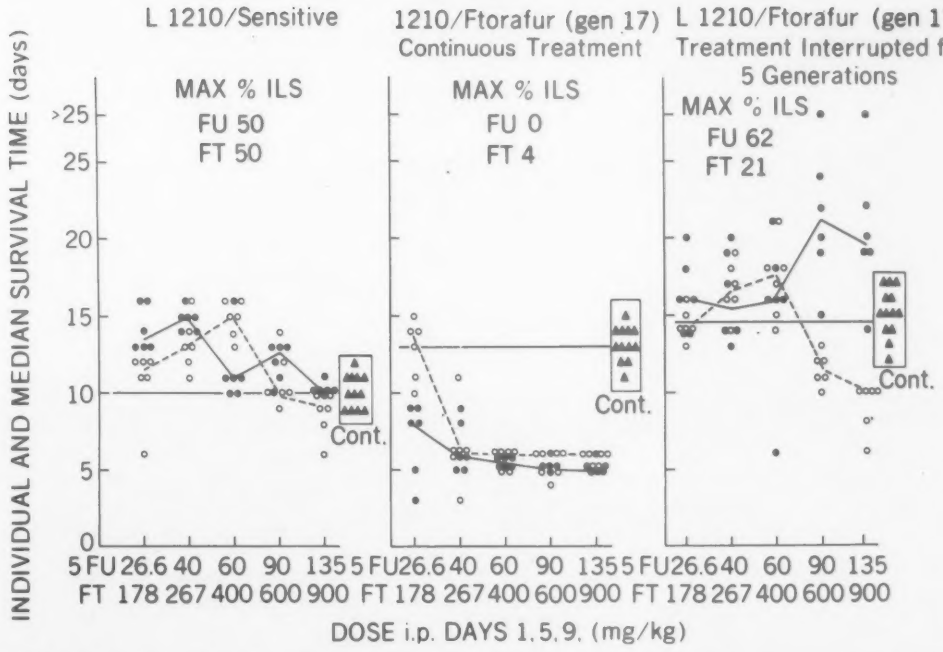


FIGURE 6.—Effects of 5-FU and ftorafur on the lifespan of mice bearing ip L1210 leukemia (L1210/sensitive), a subline of L1210 leukemia exposed to ftorafur over 17 transplant generations, and a subline exposed to ftorafur over 9 transplant generations and subsequently transplanted 8 generations in the absence of ftorafur.

ple doses there was a much greater difference in potency in killing both the hematopoietic and leukemic stem cells.

5-FU and ftorafur retained activity against L1210 leukemia on oral and sc administration. Previous comparisons of these agents in experimental tumors had shown activity on both oral (17) and parenteral administration (16). Ftorafur has been shown to be effective in man by iv (8,10–12) and oral administration (42), as well as by rectal suppository (43). Activity against ic implanted L1210 leukemia (table 2) supports pharmacokinetic (6) and clinical (8) evidence that ftorafur passes the blood-brain barrier in significant concentrations and might be useful in central nervous system tumors.

5-FU and ftorafur had a similar effect on the incorporation of uridine into RNA and deoxyuridine into DNA in L1210 leukemia cells in vivo. The inhibition of deoxyuridine incorporation was rapid in onset and persisted for several days following a single dose of the drugs. This suggests that both drugs yield effective levels of FdUMP, the proximate inhibitor of thymidylate synthetase (44), and that this anabolite persists in L1210 cells for long periods after administration of ftorafur as has also been demonstrated following administration of 5-FU (45). The inhibition of uridine incorporation was neither as complete nor as persistent as the effects on deoxyuridine incorporation. At the dose levels used, ftorafur produced a greater inhibition of uridine incorporation than 5-FU, possibly due to the prolonged release of 5-FU from ftorafur. This might explain the lower therapeutic activity of ftorafur relative to 5-FU. Greater inhibition of RNA synthesis might result in a block in the G_1 phase of the cell cycle (46) which would prevent cells from entering S phase and being killed by the inhibition of DNA synthesis at the level of thymidylate synthetase. Fujimoto et al (47) studied the effects of ftorafur on thymidine and uridine incorporation in Ehrlich ascites carcinoma in vivo. They showed an inhibition of uridine incorporation of approximately the same duration observed in the present study and a transient stimulation of thymidine incorporation (which would be expected in the presence of an inhibitor of thymidylate synthetase). Inhibition of thymidylate synthetase, as evidenced by elevated thymidine incorporation, lasted for about 12 hours in the Ehrlich ascites cells (47) as opposed to at least 96 hours in L1210 cells as shown by inhibition of deoxyuridine incorporation in the present study.

The spectrum of activity of 5-FU and ftorafur against experimental tumors was not identical (table 3). The two analogs were active against L1210

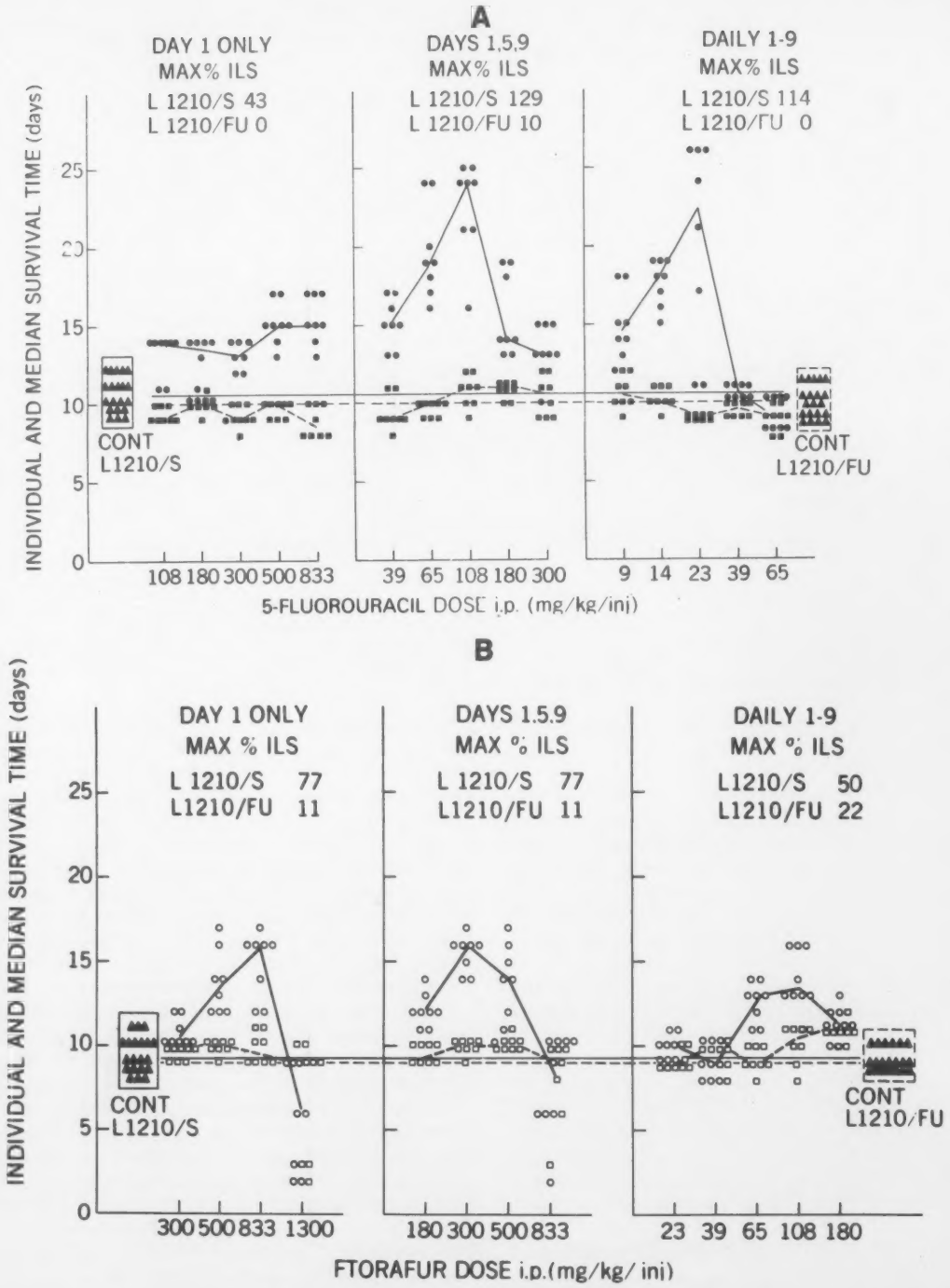


FIGURE 7.—Effects of 5-FU (A) and ftorafur (B) administered on 3 treatment schedules on the lifespan of mice bearing ip L1210 leukemia (L1210/sensitive) and a subline of L1210 leukemia exposed to 5-FU over 20 transplant generations (L1210/5-FU). ▲ = untreated controls; ●—— ● = L1210/sensitive; □----- □ = L1210/5-FU. Each symbol represents an individual mouse. The solid and dashed lines are drawn through the MST at each dose level for the sensitive and resistant sublines respectively.

leukemia and ip B16 melanoma. 5-FU was the superior drug in both of these systems. Both analogs were inactive against P1534 leukemia, sc B16 melanoma, and im Lewis lung carcinoma. Ftorafur was inactive against P388 leukemia, a tumor which was quite sensitive to 5-FU. Pallavicini and Cohen (18) found both drugs to be ineffective against P388 leukemia. This is an unusual finding for 5-FU which is used as the positive control for P388 leukemia in the large-scale screening program of the NCI (35). The Gardner 6C3HED lymphosarcoma was very sensitive to 5-FU administered ip but much less sensitive when the drug was not administered directly to the tumor cells. Ftorafur was inactive when administered ip and had only borderline activity when administered sc. It is suggested from the route dependency of 5-FU that the Gardner lymphosarcoma tumor is most sensitive when exposed to the high concentrations of 5-FU that would be achieved when the drug is administered directly into the tumor. It is not surprising, then, that ftorafur had low activity since high concentrations of the active metabolite, 5-FU, would not be achieved due to the relatively slow metabolism of ftorafur.

Combination chemotherapy studies in L1210 leukemia revealed that ftorafur plus 5-FU was no better than 5-FU alone, which supports the contention that the analogs have a similar mechanism of action. Fujimoto et al (48) concluded that 5-FU and ftorafur were therapeutically synergistic in combination against Ehrlich ascites carcinoma. However, they did not compare the combination to maximally tolerated doses of the individual agents. In combination with other agents ftorafur proved to be no better than or inferior to 5-FU. 5-FU was therapeutically synergistic in combination with methotrexate against L1210 leukemia (fig 2), confirming previous studies (49). Ftorafur plus methotrexate was no better than methotrexate alone in this tumor model. Neither 5-FU nor ftorafur was therapeutically synergistic with two agents which bind to DNA, actinomycin D and adriamycin.

The cross-resistance studies support the contention (50) that ftorafur exerts its antitumor effects primarily by elaboration of 5-FU. The emergence of resistance to ftorafur in L1210 leukemia in vivo was paralleled by the appearance of cross-resistance to 5-FU (fig 3). Similarly, a subline of L1210 leukemia with acquired resistance to 5-FU was also cross-resistant to ftorafur. These findings are in agreement with observations in microbiologic systems in which *Escherichia coli* variants resistant to 5-FU or FUdR were cross-resistant to ftorafur.[12]

[12]Pittillo RF. Personal communication.

Deletion of the enzyme(s) which activates ftorafur by cleavage of the pseudonucleoside bond to yield 5-FU could be expected to result in a cell resistant to ftorafur but not to 5-FU. Enzymatic activation of ftorafur is widely distributed in host tissues, particularly liver (2), and results in appreciable plasma levels of 5-FU after administration of ftorafur in a number of species, including man (4,6,7,34). Decreased conversion of ftorafur to 5-FU within tumor cells is thus not a likely mechanism of resistance to ftorafur and must be ruled out in the present study since cross-resistance to 5-FU was observed.

The recovery of sensitivity to 5-FU in the ftorafur-resistant subline, in which ftorafur treatment had been discontinued, occurred even though complete resistance to ftorafur was still evident (figs 4 and 5). A possible explanation for this finding could be advanced as follows. Resistance to ftorafur could likely be due to deletion of one of the enzymes involved in the anabolism of 5-FU to FdUMP, namely uridine phosphorylase, pyrimidine phosphoribosyl transferase, or uridine kinase, as has repeatedly been observed in 5-FU-resistant cells (51). After discontinuation of ftorafur treatment, it is possible that the deleted enzyme could return slowly, over several transplant generations, to levels that are present in the L1210/sensitive tumor line. Sensitivity of the tumor cells to 5-FU would reappear earlier (ie, when the enzyme levels had only partially recovered), than would sensitivity to ftorafur by virtue of the high concentrations of 5-FU to which the tumor cells are exposed immediately after a dose of 5-FU. Sufficient FdUMP would be formed to inhibit thymidylate synthetase and result in cytotoxicity. Ftorafur, on the other hand, would still appear to be ineffective because its slow metabolism would not provide the high concentrations of 5-FU necessary for production of cytotoxic levels of FdUMP. The partial return of sensitivity to ftorafur of the ftorafur-resistant subline eight transplant generations after discontinuation of exposure to the drug (fig 6) supports the contention that there is a gradual return of a deleted enzyme to levels present in the sensitive tumor line. Other explanations such as multiple selection during development of ftorafur-resistance, or the possibility that ftorafur and 5-FU have fundamentally different mechanisms of action, would seem less likely.

The peculiar pattern of enhanced toxicity to the host of both ftorafur and 5-FU was observed in the mice bearing the ftorafur-resistant line (continually treated) but not in mice bearing the 5-FU- or the ftorafur-resistant line in which treatment had been discontinued. This phenomenon is difficult to explain and is worthy of further study. Although bacterial contamination was not found, it is possible

that the ftorafur-resistant line could have been infected with a pleuropneumonia-like organism or a virus. However, the untreated controls implanted with the ftorafur-resistant line did not lose weight or show any other evidence of infection. It is possible that the enhanced toxicity might be due to enhanced metabolism of the drugs to a toxic product by the resistant tumor cells or to drug-induced elaboration of a tumor-cell related toxin.

The prolonged survival time of untreated control mice implanted with the ftorafur-resistant subline in which ftorafur treatment was discontinued is possibly due to antigenic changes in the tumor cells induced by drug treatment. Such changes have been associated with resistance to a number of antineoplastic agents (52–54), including 5-FU (55).

The reciprocal cross-resistance of ftorafur and 5-FU in this experimental tumor in conjunction with the available evidence on the similarity of their mechanisms of action (33,50) suggests that patients previously treated with and refractory as the result of biochemical resistance to one of the analogs should not be treated with the other.

Based on these studies and most previous comparisons of the therapeutic activity of 5-FU and ftorafur in experimental tumor systems (16,17), there is no evidence that ftorafur is more effective or even as effective as 5-FU. The only indication of superior activity of ftorafur was reported by Pallavicini and Cohen (18) in two transplantable mammary tumors in mice. The advantage gained by the use of this analog rests with the decreased myelosuppressive activity observed in clinical trials (8–12). This advantage would not have been predicted from studies in mice (7,56).

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Amino Acid-Conferred Resistance to Melphalan. I. Structure-Activity Relationship in Cultured Murine L1210 Leukemia Cells[1,2]

David T. Vistica, Jane N. Toal, and Marco Rabinovitz[3,*]

SUMMARY

Melphalan cytotoxicity to murine L1210 leukemia cells in culture was reduced in growth medium containing amino acids. Investigation of the effect of single amino acids revealed that the L-isomers of glutamine and leucine, but not the D-isomers, were the most active in decreasing cytotoxicity. Protection was concentration dependent, with maximum protection occurring at approximately 0.25 mM, a physiologic concentration. The LD90 for melphalan in the presence of 0.1-mM L-glutamine or L-leucine was increased by 7.3- and 10.8-fold respectively, under conditions where the cells had been pre-incubated with the amino acids. These results are interpreted to suggest that melphalan transport by the L1210 leukemia cell is mediated by a system also responsible for the transport of glutamine and leucine and that interaction with such a system may play a significant role in the chemotherapeutic activity of this alkylating agent.

[Cancer Treat Rep 60:1363–1367, 1976]

Melphalan has a broad range of antitumor activity in experimental animals (1) where single injections are curative in 60% of the mice bearing murine L1210 leukemia.[4] In man it is used in the treatment of multiple myeloma (2,3), ovarian carcinoma (4,5), breast cancer (6), and malignant melanoma (7). Although melphalan was originally synthesized with the hope that attachment of an amino acid to the nitrogen mustard group would enhance its antitumor specificity (8–10), little investigation into factors which influence its incorporation into neoplastic cells has been undertaken.

During the early course of this work it was found that L1210 cells were refractory to melphalan in growth medium containing amino acids. This prompted investigation on the specificity of this interaction and, in this communication, we present evidence that two of the naturally occurring amino acids, leucine and glutamine, are principally responsible for the decreased cytotoxicity.

MATERIALS AND METHODS

Dialyzed fetal calf serum and bovine serum albumin (BSA) (fraction V) were obtained respectively from Grand Island Biological Co, Grand Island, NY, and Miles Laboratories, Inc, Elkhart, Ind. Fetal calf serum was purchased from Flow Laboratories, Rockville, Md, and RPMI 1630 medium and Dulbecco's phosphate buffered saline (PBS) were supplied by the NIH Media Unit. Gentamicin (Schering) (50 mg/ml) was obtained from Microbiological Associates, Bethesda, Md. All amino acids were purchased from Calbiochem, San Diego, Calif.

Melphalan was prepared fresh daily as a stock solution (0.01–0.10 M) in 75% ethyl alcohol containing an equimolar concentration of hydrochloric acid.

Dialyzed fetal calf serum was replaced in the standard incubation system described below by BSA because the cytocidal effect of melphalan was found to vary considerably among different lots of serum. BSA was dissolved in Dulbecco's PBS at a concentration of 70 mg/ml and the pH adjusted to 7.4 with

[1]Received May 17, 1976; revised Aug 4, 1976; accepted Sept 1, 1976.

[2]Melphalan: NSC-8806; CAS reg. No. 3223-07-2; alanine, 3-[p-[bis(2-chloroethyl)amino]phenyl]-, monohydrochloride, L-. Purchased from Burroughs Wellcome Co, Research Triangle Park, NC.

[3]Laboratory of Medicinal Chemistry and Biology, Drug Research and Development Program (DR&DP), Division of Cancer Treatment (DCT), National Cancer Institute (NCI), Bethesda, Md.

[4]Data from the files of the DR&DP (formerly the Cancer Chemotherapy National Service Center), DCT, NCI.

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NaOH. This stock solution was sterilized by filtration, stored at 4°C, and used at a final concentration of 0.1 mM in PBS.

Cell Culture

Murine L1210 leukemia cells were grown in RPMI 1630 medium supplemented with 20% fetal calf serum and 40 μg/ml of gentamicin. Stock cultures were transferred every 2–3 days and the cell number was determined on a Coulter Counter.

Standard Incubation System—Exposure of L1210 Cells to Melphalan

Stationary phase cells (approximately 2.0–2.5 × 10^6 cells/ml) were centrifuged at 300 × *g* for 5 minutes and washed three times in one of the following media: (*a*) PBS supplemented with 0.1-mM BSA; (*b*) PBS supplemented with 10% dialyzed fetal calf serum; (*c*) RPMI 1630 medium supplemented with 10% fetal calf serum; or (*d*) RPMI 1630 medium supplemented with 0.1-mM BSA. Washed cells were suspended in Falcon 25-cm^2 flasks at a final concentration of 1.0–1.2 × 10^5 cells/ml in the appropriate medium at 37°C and melphalan or an equivalent volume of 75% ethyl alcohol was added to the appropriate cultures. The final ethanol concentration was 0.75 μl/ml. Cells were harvested after a 35-minute exposure to melphalan or the solvent, ethanol, washed three times in RPMI 1630 medium supplemented with 20% fetal calf serum, and suspended in the same medium at 1.0–1.2 × 10^5 cells/ml. The cytotoxic effect of melphalan was assessed by clonal growth.

Clonal Growth

The viability of L1210 cells was determined according to the procedure of Chu and Fischer (11) with minor modifications. DIFCO noble agar was suspended in distilled water at a concentration of 40 mg/ml, autoclaved at 120°C and 15–20 pounds per square inch for 15 minutes, and then added to growth medium kept at 45°C to give an agar concentration of 0.133%. Three ml of this nutrient-agar was dispensed at 45°C into Falcon 12 × 75-mm tubes and allowed to equilibrate at 37°C. Tenfold dilutions (10^5–10^2 cells/ml) were prepared from control and drug-treated cell populations and 1 ml of the appropriate dilution was added to the Falcon tubes containing 3 ml of nutrient-agar. The tubes were inverted, placed in an ice bath for 15 minutes, and kept at room temperature for 45 minutes. Colonies were scored after 14 days of growth at 37°C. All data are corrected for the cloning efficiency (90%–100%) of L1210 cells washed in the appropriate control medium and receiving 0.75 μl/ml of ethanol.

Amino Acid Protection of L1210 Cells Against a Melphalan LD100[5]

L1210 cells were washed and suspended in 25-cm^2 flasks with PBS containing 0.1-mM BSA and incubated for 15 minutes with the appropriate amino acid at the desired concentration. Such a pre-incubation was utilized both in order to approximate usual incubation conditions and to permit saturation of transport sites for maximum protective effect. Melphalan (2.0 μg/ml) was then added to each flask and the incubation continued for an additional 35 minutes. Melphalan toxicity was reduced by the addition of L-leucine to give a final concentration of 2 mM, and the cells were harvested, washed in growth medium, and prepared for clonal growth as described earlier.

RESULTS

Susceptibility of cultured L1210 leukemia cells to melphalan is dependent upon the composition of the incubation medium in which the cells are suspended (table 1). Dosages of melphalan which are lethal to 100% of L1210 cells suspended in Dulbecco's PBS supplemented with either BSA or dialyzed fetal calf serum are considerably less effective when cells are incubated in RPMI 1630 medium supplemented with either fetal calf serum or BSA.

Significant protection of L1210 cells against melphalan (LD100) by naturally occurring amino acids at 0.1-mM concentration is restricted to L-leucine and L-glutamine and is stereospecific (table 2). Increasing concentrations of other amino acids resulted in varying amounts of protection against melphalan; L-phenylalanine was the most effective at a 1-mM concentration (table 2). Increasing concentrations of L-leucine are accompanied by increased protection against melphalan and equimolar concentrations of L-glutamine offer less protection to L1210 cells from the cytotoxic action of melphalan (fig 1). The LD90 for melphalan was increased in the presence of 0.1-mM L-glutamine and L-leucine approximately 7.3- and 10.8-fold respectively (fig 2).

[5]LD100 is defined as the lowest concentration of melphalan (2.0 μg/ml) which consistently results in 100% cell mortality after incubation for 35 minutes in PBS supplemented with 0.1-mM BSA. This concentration is sufficiently removed from the sharp dose-response curve so as to serve as a suitable starting point for protection experiments. Cell mortality was estimated by clonal growth.

TABLE 1.—Role of the incubation medium in conferring resistance to melphalan in cultured L1210 leukemia cells*

Medium variation	Colony-forming cells/ml
RPMI 1630 medium + 10% fetal calf serum	62,600
RPMI 1630 medium + 0.1-mM BSA	36,970
Dulbecco's PBS + 10% dialyzed fetal calf serum	0
Dulbecco's PBS + 0.1-mM BSA	0

*L1210 cells (1.0×10^5 cells/ml) were incubated in the appropriate medium for 35 minutes in the presence of melphalan (2.0 μg/ml). Cultures were harvested and prepared for clonal growth as described in Materials and Methods.

TABLE 2.—Amino acids protecting L1210 leukemia cells against an LD100 dose of melphalan*

	Colony-forming cells/ml at amino acid concentration of—			
	1.0×10^{-4} M	2.5×10^{-4} M	5.0×10^{-4} M	1.0×10^{-3} M
L-Valine	0	3	21	41
L-Methionine	0	11	102	498
L-Tyrosine	0	31	364	3230
L-Phenylalanine	11	140	1459	5342
L-Isoleucine	20	261	770	3051
L-Glutamine	370	19,400	22,100	23,050
L-Leucine	10,600	34,050	37,060	40,100
D-Leucine	0	15	98	255
D-Glutamine	0	0	0	0

*L1210 cells (1.0×10^5 cells/ml) were incubated for 15 minutes in Dulbecco's PBS containing 0.1-mM BSA and the appropriate amino acid at the desired concentration. Melphalan (2.0 μg/ml) was added and the incubation continued for an additional 35 minutes. Cells were prepared for clonal growth as described in Materials and Methods. Data are expressed as the number of colonies observed after 14 days of clonal growth. The following amino acids offered no protection at 0.1 mM against an LD100 dose of melphalan: glycine, L-alanine, L-valine, L-serine, L-threonine, L-aspartic acid, L-asparagine, L-glutamic acid, L-lysine, L-arginine, L-histidine, L-tyrosine, L-tryptophan, L-cysteine, L-methionine, L-proline, L-hydroxyproline, D-leucine, and D-glutamine.

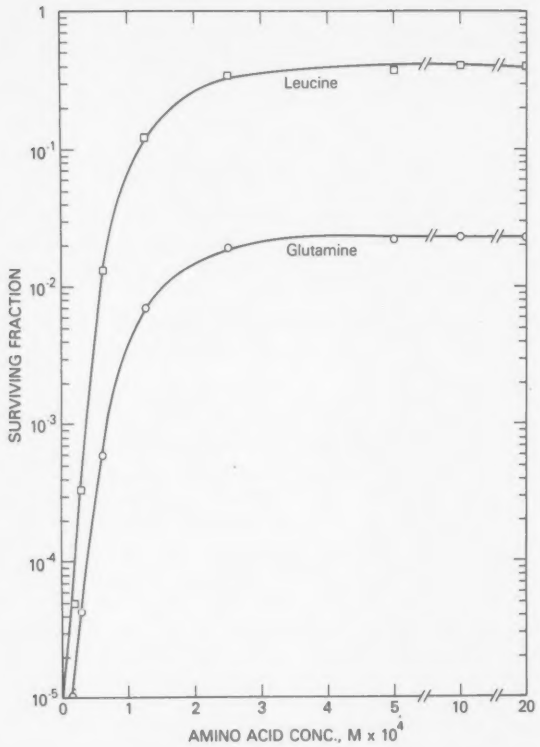

FIGURE 1.—L-Leucine and L-glutamine concentrations conferring resistance to melphalan. L1210 cells (10^5 cells/ml) were incubated for 15 minutes in Dulbecco's PBS containing 0.1-mM BSA and either L-leucine or L-glutamine at the desired concentration. Melphalan (2.0 μg/ml, an LD100 dose) was added and the incubation continued for an additional 35 minutes. Cells were prepared for clonal growth as described in Materials and Methods.

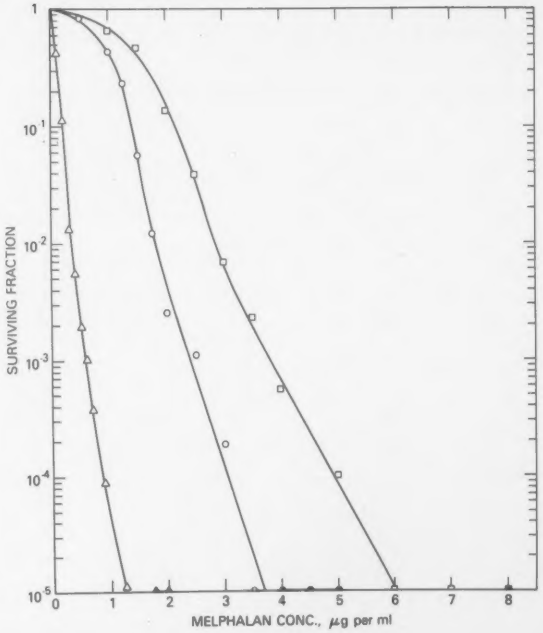

FIGURE 2.—Effect of L-leucine and L-glutamine upon the cytotoxicity of various concentrations of melphalan. L1210 cells (10^5 cells/ml) were incubated for 15 minutes in Dulbecco's PBS containing 0.1-mM BSA or in Dulbecco's PBS-BSA containing either L-leucine or L-glutamine (1.0×10^{-4} M). Melphalan was then added and the incubation continued for an additional 35 minutes. Cells were prepared for clonal growth as described in Materials and Methods. Dulbecco's PBS supplemented with 0.1-mM BSA (△—△); with 0.1-mM BSA and 0.1-mM L-glutamine (○—○); and with 0.1-mM BSA and 0.1-mM L-leucine (□—□). Open symbols on the abscissa indicate 1 cell capable of producing a colony, closed symbols indicate 100% cell mortality.

DISCUSSION

We believe that the results are best interpreted by a mechanism in which melphalan toxicity is mediated by a system responsible for the transport of amino acids. The L-isomers of leucine and glutamine are most effective in decreasing the toxicity of melphalan; the D-isomers of these two amino acids are ineffective and also have been shown to be poorly transported into mammalian cells (12,13). Work in this laboratory (unpublished observations) with the D-isomer of phenylalanine mustard, medphalan, indicates that its LD90 is approximately tenfold greater than that observed with melphalan and suggests a decreased ability of L1210 cells to transport the D-isomer of melphalan. These observations on the stereospecificity of both activity and protection, together with the pattern of protection observed with other amino acids, are most compatible with a mechanism of protection involving competition for an amino acid transport system, as opposed for example, to a direct chemical interaction of melphalan with amino acids or with inhibition of the yet more highly specific protein synthesis system.

The very low concentration of melphalan required for cytotoxicity (6.6 μM) and the protection afforded by leucine suggest the possible participation of a leucine-preferring, high-affinity, amino acid transport system. Such systems have been reported in monolayers of 3T3 cells at subconfluent culture conditions and in suspension cultures of these cells transformed by SV40 or polyoma viruses (14); they have also been studied in the Ehrlich ascites tumor cell (13).

Protection of neoplastic cells for short periods of time from the action of nitrogen mustards reduces

the therapeutic efficacy of these compounds, since they are rapidly hydrolyzed to the inactive bis(hydroxyethyl)amine derivatives (15). It therefore may be possible, by examining the amino acid protection profile of a variety of normal and neoplastic cells, to differentially protect the host by the appropriate combination of amino acids while allowing selective interaction of melphalan with tumor cells.

The above working hypothesis, that melphalan cytotoxicity is mediated through a high-affinity, leucine-preferring, amino acid transport system, and that such a system may be manipulated to chemotherapeutic advantage, will be the subject of further study.

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Phase I–II Study of DTIC and Cyclocytidine in Disseminated Malignant Melanoma[1,2,3]

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SUMMARY

Seventeen patients with disseminated malignant melanoma were treated with DTIC (150 mg/m², Days 1–5) and cyclocytidine (increasing doses sc, Days 1–10) in a phase I–II study. There was one early death. The remaining 16 patients were evaluable for response and toxicity. Two patients (13%) had CR lasting 8+ and 2+ months, while one patient (6%) had a PR lasting 1 month. Nausea and vomiting was seen in seven patients (44%), jaw pain in four (25%), and orthostatic hypotension in two (13%). Hematologic toxicity was not excessive, nor was it cumulative. The overall response rate of 19% was comparable to that reported with DTIC alone. This drug combination does not appear to offer any therapeutic advantage within the dosage range tested in disseminated malignant melanoma.

[Cancer Treat Rep 60:1369–1371, 1976]

DTIC has been shown in several studies to be the single most active drug in the treatment of disseminated malignant melanoma, producing response rates in the range of 12.5%–25% (1–4). Various drug combinations with DTIC have not resulted in significant improvement in response rate over that seen with DTIC alone. DTIC and BCNU produced a response rate of 20% (5), while BCNU, hydroxyurea, and DTIC produced a response rate of 27% (6).[5] The addition of BCG in a more recent study did not improve response rate or duration of survival (7).

Cyclocytidine is an anhydro derivative of cytosine arabinoside (ara-C), yielding ara-C upon hydrolysis, and is not subject to in vivo deactivation through deamination.[6] Because of this resistance to cytidine deaminase and other metabolic enzymes, it potentially offers more sustained blood levels, and hopefully a greater chemotherapeutic effect, than a single injection of ara-C (8). Indeed, studies by Ho et al (9) comparing different routes of administration of cyclocytidine found that iv, im, or sc routes were equally effective. With iv injections of ara-C, plasma levels were no longer detectable at 4 hours. In contrast, when cyclocytidine was given iv, plasma levels of ara-C fell exponentially with levels still detectable at 24 hours, simulating a continuous infusion. Cyclocytidine has been shown to be active in Ehrlich ascites sarcoma, Adenocarcinoma 755, ascites Sarcoma 180, and CE-1498 leukemia, and the therapeutic index is greater than that observed for ara-C in animal ascites tumors (10,11). Since Hart et al (12) reported a 23% objective response rate in metastatic malignant melanoma utilizing a 5-day continuous infusion schedule of ara-C, it seemed reasonable to combine both DTIC and cyclocytidine in a phase I–II study in patients with disseminated malignant melanoma.

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[2]Supported by Public Health Service grants CA-07127 and CA-08096 from the National Cancer Institute (NCI), National Institutes of Health, Department of Health, Education, and Welfare.

[3]DTIC: NSC-45388; CAS reg. No. 4342-03-4; imidazole-4-carboxamide, 5-(3,3-dimethyl-1-triazeno)-.

Cyclocytidine: NSC-145668; CAS reg. No. 10212-25-6; 6*H*-furo[2′,3′:4,5]oxazolo-[3,2-*a*]pyrimidine-2-methanol, 2,3,3*a*,9*a*-tetrahydro-3-hydroxy-6-imino-, monohydrochloride.

Both drugs were supplied by the Cancer Therapy Evaluation Program, Division of Cancer Treatment, NCI.

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[5]BCNU: NSC-409962; CAS reg. No. 154-93-8; urea, 1,3-bis(2-chloroethyl)-1-nitroso-.

Hydroxyurea: NSC-32065; CAS reg. No. 127-07-1.

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[6]Cytosine arabinoside: NSC-63878; CAS reg. No. 69-74-9; cytosine, 1-β-D-arabinofuranosyl-, monohydrochloride.

MATERIALS AND METHODS

Seventeen patients (12 women, five men) with disseminated malignant melanoma were entered in this study. Two patients had received prior BCG, one had received prior DTIC and adriamycin, and the remaining 14 had had no prior therapy except surgery. Patients were evaluated before therapy with regard to tumor measurements as well as enzymatic and hematologic parameters. Sixteen patients were evaluable for toxicity and response. One patient died 3 days after the initiation of therapy and was considered unevaluable. All patients except two had extensive extranodal disease, including brain, lung, and/or liver metastases. The median age of the patients was 51 years (range, 29–76 years).

DTIC was administered iv over 15–20 minutes at a dose of 150 mg/m²/day, Days 1–5 for all patients. Cyclocytidine was given sc daily on Days 1–10. Four patients were treated at a dose of 100 mg/m², three at 150 mg/m², five (four evaluable) at 225 mg/m², and five at 270 mg/m². Courses were repeated at 4-week intervals providing there was hematologic recovery (wbc count > 3000 cells/mm³, platelet count > 100,000 cells/mm³). The 16 patients received a total of 28 courses of chemotherapy. A patient was considered to have received an adequate trial if two or more courses of therapy were received or increasing disease (> 25%) was noted after one course of therapy.

CR was defined as the disappearance of all clinical evidence of active tumor for a minimum of 4 weeks together with a disappearance of all tumor-related symptoms. PR was defined as a > 50% decrease in the sum of the products of the largest perpendicular diameters of all measured lesions lasting at least 4 weeks in the absence of significant increase in any one lesion or the appearance of new lesions. Disease progression was defined as unequivocal increase of any measured lesion or the appearance of new lesions.

RESULTS

Response

Of the 17 patients entered in the study, one died 3 days after initiation of therapy and was considered unevaluable for response and toxicity because of early death. Of the remaining 16 evaluable patients, two (13%) had a CR lasting 8+ months (treated at doses of 150 and 225 mg/m² of DTIC and cyclocytidine respectively) and 2+ months (treated at doses of 150 and 270 mg/m² of DTIC and cyclocytidine respectively), and one (6%) had a PR (treated at doses of 150 and 270 mg/m² of DTIC and cyclocytidine respectively) lasting 1 month for an overall response rate of 19%. The remaining 13 patients (81%) had clear-cut evidence of disease progression. One complete responder had disease in the retroperitoneal space, lungs, and pleura while in the other, disease was confined to the subcutaneous tissue. The one partial responder had disease in skin, lymph nodes, and lung.

Toxicity

Nonhematologic toxicity could be evaluated in all 16 patients. Nausea and vomiting was present in seven patients (44%) and although not mentioned, was not noted to be absent in the other nine. Jaw pain was seen in four patients (25%) while orthostatic hypotension was seen in two (13%). The above-mentioned toxicities were not excessive or debilitating (ie, they did not require iv supplementation, hospitalization, etc) and were adequately controlled with appropriate medications. Hematologic toxicity was evaluated in all 16 patients. Twenty-eight courses were given and in no instance was there delay in administering second or subsequent levels of therapy because of failure to recover from bone marrow suppression. Cumulative toxicity was not observed. For all dose ranges (table 1) the nadir of thrombocytopenia preceded the nadir of leukopenia but with full recovery by Day 29. No patient required hospitalization for infectious or hemorrhagic complications of myelosuppression nor were there any drug-related deaths.

DISCUSSION

Cyclocytidine, a synthetic analog of ara-C, has been shown by Ho et al (9) to behave pharmacokinetically in a similar fashion to continuous ara-C infusion. Since Hart et al (12) had shown a 23% response rate to ara-C infusion in patients with disseminated malignant melanoma, it seemed reasonable to combine cyclocytidine with DTIC, the most effective single agent against malignant melanoma.

While the combination was well-tolerated without life-threatening toxicity, the overall response rate of 19% was comparable to that reported with DTIC alone. Further dose escalations were not attempted, since responses were seen at the previously mentioned dosage levels. We feel that further clinical trial of this drug combination in disseminated malignant melanoma, within the dosage range tested, is not warranted.

TABLE 1.—Hematologic toxicity

Doses of DTIC/cyclocytidine (mg/m²)	No. of patients treated	No. of courses	Wbc count		Platelet count	
			Nadir (× 10^3 cells/mm³)	No. of days to nadir	Nadir (× 10^3 cells/mm³)	No. of days to nadir
150/100	4	6	Mean, 6.2 Median, 5.8 Range, 3.6–9.8	19.5 20 13–25	Mean, 105 Median, 88 Range, 78	15.3 15.5 13–17
150/150	3	6	Mean, 6.1 Median, 7.3 Range, 3.1–7.9	26.3 26 22–31	Mean, 196 Median, 209 Range, 105–275	19.3 14 13–31
150/225	4	10	Mean, 3.4 Median, 3.2 Range, 2.2–4.8	24.7 26 19–28	Mean, 169 Median, 180 Range, 49–264	26 23.5 16–41
150/270	5	6	Mean, 3.7 Median, 3.1 Range, 1.5–6.0	21 20 10–29	Mean, 158 Median, 118 Range, 105–245	15 15 10–20
Totals	16	28				

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Combination Chemotherapy With Mitomycin C, 5-Fluorouracil, and Cytosine Arabinoside in Gastrointestinal Cancer[1,2,3]

Brief Reports and Preliminary Communications

Robert L. De Jager, Gordon B. Magill, Robert B. Golbey, and Irwin H. Krakoff[4,*]

The combination of mitomycin C, 5-fluorouracil (5-FU), and cytosine arabinoside (ara-C) (MFC) was reported by Hoshino et al (1) to have a marked synergistic effect in the treatment of L1210 mouse leukemia. Subsequently, Ota et al (2) reported a response rate of 55% in patients with stomach cancer and 60% in patients with colon cancer. The purpose of this study was to evaluate further the efficacy of MFC in the treatment of gastrointestinal malignancies.

METHODS

MFC was given to patients with advanced, histologically confirmed, inoperable or recurrent carcinoma of the stomach, pancreas, gallbladder, liver, and unknown primary. At least one measurable parameter of disease and an expected survival of 1 month were required. The extent of disease was determined before treatment and at appropriate intervals with the relevant clinical laboratory, radiographic, and isotopic techniques.

Mitomycin C (0.04–0.08 mg/kg), 5-FU (7.5–10 mg/kg), and ara-C (0.6 or 2.0 mg/kg) were given iv twice a week until hematopoietic toxicity occurred. There was considerable variability in the number of doses tolerated. After the appearance of hematopoietic toxicity, treatment was repeated once or twice a week as permitted by blood cell counts. After a small initial group of patients were treated, the dose of ara-C was increased from 0.6 mg/kg (the dose used by Ota et al) to what we believed to be a biologically more effective dose level (2.0 mg/kg). The actual doses are indicated in table 1. Treatment continued until disease progression occurred.

Responses were classified as: *complete response* (CR)—disappearance of all signs and symptoms of disease for a period of ≥ 1 month; *partial response* (PR)—reduction in the product of two diameters of all measurable lesions by ≥ 50% for a period of ≥ 1 month; *minor response*—any response lasting < 1 month or a reduction in the product of two diameters of some or all measurable lesions of < 50%. Minor response, no change, or progression of any measurable lesions during therapy were considered as no response (NR).

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[2]Supported in part by Public Health Service grants CA-05826 and CA-08748 from the National Cancer Institute, National Institutes of Health, Department of Health, Education, and Welfare.

[3]Mitomycin C: NSC-26980; CAS reg. No. 50-07-7.

Cytosine arabinoside: NSC-63878; CAS reg. No. 69-74-9; cytosine, 1-β-D-arabinofuranosyl-, monohydrochloride.

5-Fluorouracil: NSC-19893; CAS reg. No. 51-21-8.

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TABLE 1.—MFC clinical results–relation of response to dose of ara-C

Tumor type	Total No. of patients treated	Ara-C (0.6 mg/kg) No. of patients treated	Ara-C (0.6 mg/kg) PR	Ara-C (2.0 mg/kg) No. of patients treated	Ara-C (2.0 mg/kg) PR
Carcinoma of—					
Stomach	32	6	2	26	8
Colon	37	15	1	22	2
Pancreas	9	2	0	7	0
Gallbladder/liver	5	2	0	3	0
Unknown primary	13	3	1	10	0

TABLE 2.—Patient characteristics (adequate trial)

Characteristics	
Total No. of patients	96
Men/women	55/41
Mean age in yrs (range)	59 (20–85)
Mean performance status (%) (range)	60 (30–90)
Prior radiotherapy	25
Prior chemotherapy:	
Total	49
1 drug	33
≥ 2 drugs	16
5-FU	47
Alkylating agent	14
Methotrexate	7
Vinca alkaloid	1
Nitrosourea	3
Actinomycin D	1

The adequacy of the trial was defined by the appearance of toxicity or by treatment of at least 1 month's duration; 96 patients received adequate trials of MFC. Patient characteristics are shown in table 2.

RESULTS

A mean of 7.5 doses (range, 3–19) were given to each patient before the appearance of hematopoietic toxic effects. Hematopoietic toxicity was dose limiting and prolonged (5–34 days), usually occurring after five treatments. The sixfold increase in the dose of ara-C did not change the level of cell count depression. Nausea and vomiting were the prominent gastrointestinal toxic effects; diarrhea and stomatitis were seen less frequently. Alopecia was rare (table 3). Table 4 summarizes the clinical responses of the tumor types treated.

The overall response rate was 15%. Although there were no CRs, there was a 31% (ten of 32 patients) PR rate in carcinoma of the stomach. The median duration of response in stomach cancer was 3 months (range, 1–10 months). The responses were clinically useful and characterized by symptomatic improvement, weight gain, and tumor regression. There was no significant difference in response rate between patients who had had no previous chemotherapy with 5-FU (seven of 21) and patients who had been previously treated with 5-FU (three of 11) (table 4). As shown in table 1, response rates were similar in patients who received high- and low-dose ara-C.

TABLE 3.—MFC toxicity

Toxic effect	% of patients
Hematopoietic	
Wbc < 4500 cells/mm³*	84
Platelets < 150,000 platelets/mm³†	83
Nausea and vomiting	47
Diarrhea	14
Stomatitis	10
Alopecia	3
Fever	1

*Mean nadir: 3000 cells/mm³ (range, 200–5200 cells/mm³).
†Mean nadir: 100,000 platelets/mm³ (range, 7000–500,000 platelets/mm³).

DISCUSSION

The MFC regimen shows definite activity in carcinoma of the stomach. This contrasts sharply with the lack of response of colon cancer and other gastrointestinal malignancies. A similar difference has been reported by Livingston and Carter (3) for mitomycin C used alone.

The similar response rate for the group previously treated with 5-FU and the group without prior treatment suggests that mitomycin C may be the major active component of this combination. This is also suggested by the report of Livingston and Carter (3) of a 32% response rate in gastric cancer with mitomycin C alone. Their report emphasizes the severe toxic potential of mitomycin C; however,

TABLE 4.—MFC clinical results—relation of response to prior 5-FU therapy

Tumor type	Total No. of patients treated	Prior 5-FU		No prior 5-FU	
		No. of patients treated	PR	No. of patients treated	PR
Carcinoma of—					
Stomach	32	11	3	21	7
Colon	37	25	1	12	2
Pancreas	9	3	0	6	0
Gallbladder/liver	5	2	0	3	0
Unknown primary	13	4	1	9	0

the toxicity from our MFC combination study was moderate, and the regimen was suitable for outpatient administration.

The role of ara-C in the MFC combination is unclear. Its activity as a single agent in solid tumors is not significant except for a report by Frei et al (4) in gastric cancer.

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Comparison of the Biologic Activity of VP-16-213 Given Iv and Orally in Capsules or Drink Ampules[1,2]

Brief Reports and Preliminary Communications

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VP-16-213 is the newest in a series of semisynthetic podophyllotoxin derivatives to exhibit antineoplastic effects in experimental tumor systems (1,2) and in man (3–8). The iv dosage and scheduling in man have been studied clinically (9,10). However, there has been little experience with oral dosage (11). The mechanism of action is initial metaphase arrest and subsequent block in the G_2 phase (12).

Our study compares the effects of VP-16-213 given iv and orally in the form of capsules or drink ampules. This was accomplished clinically by determining the biologic activity of each administration method as reflected primarily by changes in the leukocyte and platelet counts as well as other toxic manifestations.

PATIENTS AND METHODS

Patients were randomized into one of three basic treatment groups. Previous treatment or tumor type were not considered in the randomization process.

Group I patients were given 100 mg/m²/day of VP-16-213 for 3 days. This was applied as a 30-minute iv infusion. The 3-day infusion series was repeated in 8 days. In patients with leukopenia and/or thrombocytopenia, the second 3-day infusion series was delayed until the leukocyte counts had risen to > 4000 cells/mm³ and the platelet counts to > 100,000 platelets/mm³.

Group II patients received VP-16-213 in 100-mg capsules for 3 days in a dose of 200 mg/m²/day. The 3-day treatment course was repeated in 8 days if the leukocyte counts had recovered to > 4000 cells/mm³ and the platelet counts to > 100,000 platelets/mm³. Treatment was delayed when necessary until these minimal values were reached.

After at least two 3-day courses of treatment (but not before 14 days or before recovery of leukopenia to > 4000 cells/mm³ and thrombocytopenia to > 100,000 platelets/mm³) these patients were given two 3-day courses of VP-16-213 in drink ampules. The dosage here was also 200 mg/m². Again, after two 3-day courses of drink-ampule treatment and at least a 14-day interval, these patients received VP-16-213 capsules at the same dosage in 3-day courses if the minimum leukocyte values of 4000 cells/mm³ and platelet values of 100,000 platelets/mm³ were reached. In this manner a single or repeated crossover was carried out for each patient.

Group III patients received primary treatment with VP-16-213 drink ampules (200 mg/m²) daily for 3 days. After two of these 3-day courses over a minimum of 14 days and recovery of the leukocyte and platelet counts, a crossover to capsule treatment was made. Similar to the procedure for group II patients, such crossovers were repeated at 14-day intervals, or as the toxic manifestations allowed.

For this study, the same ampules used for iv treatment were employed as drink ampules. The calculated total daily dose was divided into two or three doses. It was administered in orange or grapefruit juice in an attempt to disguise the bad taste.

The primary study parameters consisted of a comparison of the depression of the leukocyte and thrombocyte counts between the various administration methods. These counts were done at least weekly. Other toxic manifestations such as hair loss, gastrointestinal effects, and stomatitis were also considered.

RESULTS

Sixteen patients had 21 courses of therapy with capsules, 16 had 23 courses of drink-ampule treatment, and 12 had 26 courses of iv infusion therapy; all of these patients were available for final evaluation.

The leukocyte nadir was determined for each individual course of treatment and expressed as a point value in one of five categories (0–4) according to the key in table 1. Calculation of the total and average point values for each type of treatment makes a direct comparison of the leukopenic effects of the three treatment forms possible. Under pri-

[1]Received Feb 11, 1976; revised Aug 30, 1976; accepted Aug 31, 1976.

[2]VP-16-213: NSC-141540; 4'-demethylepipodophyllotoxin 9-(4,6-*O*-ethylidene-β-D-glucopyranoside). Supplied by Sandoz Ltd, Basel, Switzerland.

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mary VP-16-213 capsule treatment nine of 16 patients had leukopenia, but only three of these were in categories 2 or 3. Primary treatment with VP-16-213 drink ampules resulted in a leukocyte drop in each of the nine evaluable courses of therapy. A drop in the wbc count could also be observed in all of the 26 courses of infusion therapy.

There were 13 crossovers from primary capsule application to secondary drink-ampule therapy. The average degree of leukopenia rose 1.9 point values with drink-ampule therapy above that observed under the initial capsule treatment. None of the crossovers from drink-ampule to capsule treatment gave a greater degree of leukopenia.

A direct comparison of the degree of thrombocytopenia between the three application forms shows that a slight fall in the platelet counts was observed in only four out of a total of 16 courses of primary treatment with VP-16-213 capsules. Only one value $< 100{,}000$ platelets/mm^3 was recorded. No platelet depression occurred with the secondary capsule therapy. In contrast, a greater fall in platelet counts could be observed following primary as well as secondary treatment with VP-16-213 drink ampules. The 26 courses of VP-16-213 infusion therapy resulted in a thrombocyte fall in 17 cases. In general, the degree of platelet depression was less severe than that of leukopenia in all three treatment forms.

Other significant toxic manifestations were hair loss, gastrointestinal effects such as anorexia, nausea and vomiting, and relatively rare stomatitis. In three cases moderate diarrhea was seen under secondary drink-ampule treatment after initial capsule therapy. Only hair loss, stomatitis, and gastrointestinal manifestations were evaluated in a semiquantitative manner. The incidence and degree of these toxic manifestations with drink ampules were approximately threefold that encountered with capsule treatment. Hair loss was equal with drink-ampule and infusion therapy. Gastrointestinal toxicity and stomatitis with drink ampules were almost double that with infusions.

THERAPEUTIC RESULTS

Due to the small number of patients planned for this study, it was not anticipated that significant differences in therapeutic results would evolve. There was a partial remission of $> 50\%$ seen in each treatment group. These included a thyroid carcinoma, one small cell bronchogenic carcinoma, and one anaplastic bronchogenic carcinoma.

TABLE 1.—Direct comparison of the leukocyte fall in three treatment forms

Type of administration	No. of courses	Category of leukopenia* 0	1	2	3	4	Total points†	Average points/ course‡	Day of nadir
Capsules									
Primary	16	7	6	2	1	0	13	0.8	13 (7–22)
Secondary	5	5	0	0	0	0	0	0	—
Total	21	12	6	2	1	0	13	0.6	13 (7–22)
Drink ampules									
Primary	9	0	1	3	3	2	24	2.7	14 (3–20)
Secondary	14	0	2	3	5	4	39	2.8	10 (6–19)
Total	23	0	3	6	8	6	63	2.7	12 (3–20)
Iv infusions	26	0	5	7	11	3	64	2.5	15 (7–22)

*Key for determining the category of leukocyte fall:

Cell counts (cells/mm^3) in patients with initial—	
Normal values	*Low values*
0 = no decrease	No decrease
1 = 4100–5000	Fall of 500–1000
2 = 3100–4000	Fall of 1000–2000
3 = 2100–3000	Fall of 2000–3000
4 = < 2000	Fall of > 3000

†No. of cases in each category × point value of that category.
‡Total point value divided by No. of courses.

DISCUSSION

Based upon the degrees of toxicity observed, treatment with VP-16-213 drink ampules was more effective when employed as primary treatment as well as secondary treatment after crossover from primary capsule therapy. This was noted in the leukocyte and platelet count nadirs as well as in the severity and incidence of hair loss and gastrointestinal side reactions.

Capsules and drink ampules were given in an equal dosage (200 mg/m^2/day for 3 days). The more severe toxic effects seen with the drink ampules may be explained by a better resorption of the drug. When compared with iv infusion treatment, drink-ampule therapy, given in twice the daily dosage, produced a slightly greater biologic effect as judged by degree of leukopenia, stomatitis, and gastrointestinal toxicity.

If infusion therapy with VP-16-213 represents 100% resorption, then it may be assumed that the double dose of drink ampules used had a resorption of slightly > 50%. Another possible explanation may be a greater biologic effect with a three times daily oral administration than with a once daily iv application.

Whether or not the assumed better resorption of the VP-16-213 drink ampules as compared with the capsules may be reflected in a superior therapeutic effect cannot be answered by this study. A greater degree of toxicity is not necessarily associated with a greater therapeutic effect.

Drink-ampule treatment, in the form used in this study, may well be limited by the bad taste of the substance. For more extended clinical use, it would be mandatory to have a more palatable pharmaceutical preparation available. The convenience of an active oral form of this drug, especially for use in ambulatory patients, cannot be disputed.

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Combination Cytotoxic Chemotherapy for Metastatic Cutaneous Malignant Melanoma With DTIC, BCNU, and Vincristine[1,2]

Brief Reports and Preliminary Communications

J. Carmo-Pereira,* F. Oliveira Costa, and Pedro Pimentel[3,4]

Malignant melanoma is an uncommon tumor and the treatment of this disseminated disease has been a challenging problem for the oncologist and more recently for the immunologist. In view of the effectiveness of combination cytotoxic chemotherapy for some tumors (1), a number of regimens are being used in disseminated cutaneous malignant melanoma, a disease usually regarded as relatively resistant to cytotoxic chemotherapy. The results presented by Cohen et al (2), Johnson et al (3), and Luce et al (4) on the treatment of this disease (stage IV) are encouraging and we have undertaken a similar protocol to verify these results.

PATIENTS AND METHODS

Twenty consecutive evaluable patients (age range, 32–71 years; median age, 48.5 years) with disseminated cutaneous malignant melanoma (stage IV) that was histologically proven, were studied from February 1, 1973, to November 30, 1975. Before chemotherapy, patients were evaluated for metastatic disease by physical, radiologic, and radioisotopic examination. All had a performance status of ≥ 50% (according to the Karnofsky scale), normal liver and renal function studies, no prior exposure to any of the drugs utilized in this protocol, and no previous immunotherapy. The criteria used for objective evaluation of response have been previously described by Carter (5): *complete response (CR)*—disappearance of all lesions of active tumor for > 1 month; *partial response (PR)*—≥ 50% decrease in the products of the longest diameters of all measurable lesions for > 1 month; *static disease*—changes smaller than those outlined in PR; *progression*—increase of > 25% in the size of measurable lesions or the appearance of new lesions. The duration of response was determined from remission induction to progression.

The schedule used was: vincristine, 1.4 mg/m² iv Days 1 and 8; DTIC, 250 mg/m² iv Days 1–3; and BCNU, 65 mg/m² iv Days 1–3, with a 4–6-week interval after each course.

If myelotoxicity developed, the interval between the courses was increased to but never exceeded 10 weeks, as this was always enough time for bone marrow recovery. Blood cell counts were determined at 2-week intervals and before each treatment course. The number of leukocytes had to be ≥ 4000/mm³ and the platelets ≥ 100,000/mm³ for treatment to be given. A patient was considered evaluable even if only one cycle of therapy was given. The differences in survival between responders and nonresponders were analyzed statistically using the student's t test to assess significance.

RESULTS

Antitumor Effects

The overall response rate was 35% (seven of 20 patients). Five patients achieved CRs lasting 6, 9, 11, 14, and 14 months respectively, and two patients had PRs for a duration of 6 and 15 months respectively. The median duration of remission was 11 months (range, 6–15 months). The number of courses given to each patient varied from one to nine. All patients had died by the end of the trial (November 30, 1975) and all of the seven responders had achieved a performance status of 90% or more. Of the 13 nonresponders, only two had had static disease (3 and 4 months respectively). The survival of responders was significantly longer than that of nonresponders ($P < 0.001$; *see* fig 1 and table 1).

[1]Received Apr 5, 1976; revised Aug 30, 1976; accepted Sept 2, 1976.

[2]DTIC: NSC-45388; CAS reg. No. 4342-03-4; imidazole-4-carboxamide, 5-(3,3-dimethyl-1-triazeno)-.

BCNU: NSC-409962; CAS reg. No. 154-93-8; urea, 1,3-bis(2-chloroethyl)-1-nitroso-.

Vincristine: NSC-67574; CAS reg. No. 2068-78-2; leurocristine, sulfate (1:1).

DTIC and BCNU were supplied by Dr. Stephen K. Carter of the Cancer Therapy Evaluation Program, Division of Cancer Treatment, National Cancer Institute.

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Toxic Effects

All patients experienced nausea and vomiting which lasted for the first 3 days of treatment; because of this, one patient refused further treatment after the second course. Pretreatment and subsequent use of phenothiazine derivatives helped to control this toxic effect. A burning sensation along the vein selected for injection was present in all of the patients during the administration of BCNU; this drug must be given after vincristine and DTIC because, if given first, this burning sensation also occurs during the administration of DTIC. To minimize this toxic effect, 2 ml of lydocaine was given iv immediately before the BCNU administration. Leukopenia of 1000–4000 cells/mm^3 was observed in 11 patients (55%), but counts < 1000 cells/mm^3 did not occur; thrombocytopenia of < 100,000 platelets/mm^3 was observed in five patients (25%), the lowest value being 30,000 platelets/mm^3. This hematologic toxicity was more evident after the third course of therapy at approximately the fourth week after the beginning of the cycle, probably due to the cumulative effect of DTIC and BCNU. In two patients (10%) severe gastrointestinal bleeding, purpura, and echymoses occurred 4–6 weeks after the previous treatment course, due to thrombocytopenia. These severe toxic effects were controlled by blood transfusions. In five patients (25%) a peripheral neuropathy, grade 1 (6), was detected. Alopecia did not occur and there were no drug-related deaths.

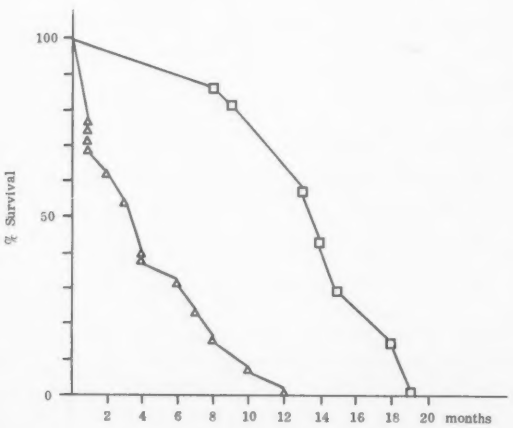

FIGURE 1.—Survival rate of melanoma patients (7 responders [□] and 13 nonresponders [△] after cytotoxic chemotherapy ($P < 0.001$).

TABLE 1.—Survival (mos)*

	Responders	Nonresponders
Mean ± SD	13.7 ± 4.2	4.6 ± 3.7
Range	8–19	1–12

*$P < 0.001$.

COMMENT

In primary melanoma, the clinical variety (3,7,8) (lentigo maligna, superficial spreading, or nodular melanoma), the degree of microinvasion (7,9), the site of the primary tumor (10), and the occurrence of disease in the regional lymph nodes (10) are the main determinants of prognosis. In disseminated melanoma, the prognosis is also related to the free interval (4), location of dominant metastatic lesions (2,11), and sex (3,4,10,12). In therapeutic trials, all these variables make an accurate evaluation difficult.

The results obtained depend on the criteria of assessment and the nature of the group of patients treated. In this trial the percent of objective remissions (35%) was different than that recorded by Cohen et al (62.5%) (2), but close to that of Beretta et al (44%) (11) and Luce et al (24%) (13), all of whom used similar protocols with the same drugs. The best results we obtained were in women and in cases with nodal and/or subcutaneous involvement; these are good-risk patients as previously noted by others (2,10–12). Our results failed to show a better response in relation to a longer free interval (4). Six of the patients with objective remission had a free interval of < 1 year. We have included and considered as evaluable five patients who received only one treatment cycle. According to the criteria of the NIH these patients should be deleted. If we adhered to this then the better results obtained by some investigators might have been confirmed. Our results demonstrate worthwhile improvement in the "good prognosis patients" and we recommend the use of this drug combination only for these patients. These therapeutic effects however, warrant further study in the treatment of this disease. Randomized studies with different combinations are now in progress in our center.

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A Comparative Clinical Trial Evaluating the Combination of Adriamycin, DTIC, and Vincristine, the Combination of Actinomycin D, Cyclophosphamide, and Vincristine, and a Single Agent, Methyl-CCNU, in Advanced Sarcomas[1]

Brief Reports and Preliminary Communications

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Polychemotherapeutic approaches for soft tissue sarcomas have included combinations of actinomycin D, cyclophosphamide, and vincristine (1). The response rate is reportedly in the range of 24%. Reports of utilizing adriamycin and DTIC in 200 patients showed a 42% response rate, and a three-drug program of DTIC, adriamycin, and vincristine produced a response in 19 of 40 patients (48%) (2). When cyclophosphamide was added, 49 of 82 patients (60%) responded with the median duration of response of > 6 months (3).

We will summarize our experience with the combination of adriamycin, DTIC, and vincristine; the combination of actinomycin D, cyclophosphamide, and vincristine, and a single agent, methyl-CCNU, in advanced sarcomas.

MATERIALS AND METHODS

Eighty patients with advanced sarcomata participated in a randomized trial comparing the chemotherapeutic programs described. There were 42 men and 38 women with a mean age of 44.2 years. Seventy-three patients (93%) had visceral-dominant disease and only five patients had received prior chemotherapy. All patients were acceptable candidates for cytotoxic chemotherapeutic drug trials with respect to performance score and hematologic status.

The combination of DTIC, adriamycin, and vincristine was administered as follows: DTIC, 250 mg/m² iv on Days 1–5; adriamycin, 50 mg/m² iv on Day 3; and vincristine, 1.2 mg/m² (not to exceed 2 mg) iv on Days 1 and 5. The alternative combination program included: actinomycin D, 325 μg/m² iv on Days 1–5; cyclophosphamide, 250 mg/m² iv on Days 1, 3, and 5; and vincristine as previously stated. Both combination programs and evaluations of response were repeated every 5 weeks from the first day of treatment. The single agent, methyl-CCNU, was given orally at a dose of 200 mg/m² every 7 weeks.

A partial regression was defined as a ≥ 50% decrease in the product of diameters of measurable lesions lasting a minimum of 4 weeks. A complete regression was defined as the disappearance of all evidence of disease. Durations of response were calculated from the start of chemotherapy until there was evidence of progressive disease. Progressive disease was defined as a > 25% increase in measurable disease or the appearance of new lesions.

If after therapy an objective regression had occurred or if disease had not progressed, treatment with the same program was continued. With evidence of objective progression, patients were randomized to the alternate treatment regimens. Methyl-CCNU was not utilized after 11 consecutive patients failed to respond.

RESULTS

After initial chemotherapy exposure, only six of 80 patients (8%) achieved the status of objective response, with a median duration of response of 6 months (table 1). Fifty patients with progressive disease crossed over to secondary treatment programs. Of these 50, five responded (10%) for a median duration of 6 months. From the start of primary and secondary treatment, the median response duration achieved with either combination program was approximately 6 months. No patients responded to methyl-CCNU (table 2).

The most frequent toxic effects from the combination programs were nausea, vomiting, and alopecia. Stomatitis occurred in 30% of the patients receiving

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TABLE 1.—Response by diagnosis and primary chemotherapy (DAV = DTIC, adriamycin, and vincristine; ACV = actinomycin D, cyclophosphamide, and vincristine; me-CCNU = methyl-CCNU)

Cell type	No. of patients	Objective responses		Objectively stable patients	
		No.	Duration (mos)	No.	Duration (mos)
Leiomyosarcoma	17	0	—	2	5(DAV), 18(ACV)
Osteogenic sarcoma	16	2	3(DAV), 7(ACV)	2	6(me-CCNU), 7(DAV)
Rhabdomyosarcoma	9	2	5(DAV), 27(DAV)	0	—
Fibrosarcoma	8	1	9(ACV)	1	10(DAV)
Hemangiopericytoma	5	0	—	1	4(ACV)
Fibrous histiocytoma	5	0	—	0	—
Mesothelioma	4	0	—	1	8(ACV)
Miscellaneous	16	1*	6(DAV)	2†	3(DAV), 10(ACV)

*Liposarcoma.
†Neurofibrosarcoma; alveolar soft part sarcoma.

TABLE 2.—Duration of response and stability by treatment programs

Agents	No. of responses							
	Primary treatment				Secondary treatment			
	No. of patients treated	Partial	Complete	Stable ≥ 3 mos	No. of patients treated	Partial	Complete	Stable ≥ 3 mos
DTIC, adriamycin, vincristine	31	2	1	4	23	3*	0	4†
Actinomycin D, cyclophosphamide, vincristine	38	2	1	4	23	2‡	0	0
Methyl-CCNU	11	0	0	1	4	0	0	0

*Osteogenic sarcoma; fibrosarcoma; synovioma.
†Hemangiopericytoma (2 patients); rhabdomyosarcoma; leiomyosarcoma.
‡Leiomyosarcoma (both patients).

actinomycin D. Approximately one half of the patients receiving vincristine noted paresthesias, but no significant motor impairment occurred. Leukopenia (wbc < 3000 cells/mm^3) occurred in 42 of 80 patients and one half of these patients had a count < 1500 cells/mm^3. Toxicity was comparable between the combination regimens. No cardiomyopathy was observed after adriamycin. There were few side effects from methyl-CCNU.

DISCUSSION

Using the combination of DTIC, adriamycin, and vincristine, an 11% response rate occurred after primary and secondary treatments. This contrasts with a reported response rate of 48% in patients with approximately the same distribution of tumors but treated with a different schedule (2); in this study adriamycin (60 mg/m^2) was given on Day 1 instead of Day 3, and the three-drug program was repeated every 3 weeks versus every 5 weeks as in our study. Nausea, vomiting, alopecia, and stomatitis were equivalent in both programs. Leukopenia (wbc < 3000 cells/mm^3) occurred in 65% of our patients compared with 86% previously reported.

After the combination of actinomycin D, cyclophosphamide, and vincristine, 8% of the patients responded to primary and secondary treatments compared with a 47% response rate previously reported (1). The doses of the individual agents were approximately the same in both studies. However, the previous series had twice as many patients with rhabdomyosarcomas, allegedly a more sensitive tumor. The interval between treatments and the number of agents used are not clear in the previous report. In our study, nausea and vomiting were three times more frequent and stomatitis was eight times more common. Leukopenia occurred in 21 of

38 patients in our series but could not be assessed from the earlier report.

There were no responders among 15 patients receiving methyl-CCNU as primary or secondary treatment. This observation is similar to a recent report of 32 patients with sarcomas treated with iv methyl-CCNU. Only two patients responded and two remained stable (4).

We were unable to demonstrate with our regimens the previously reported favorable results in utilizing polychemotherapy in advanced sarcomas. There are several possible explanations. First, adriamycin was used at a dose of 50 mg/m² every 5 weeks in our combination rather than 60 mg/m² every 3 weeks as previously reported by others (2). However, the severity of gastrointestinal and hematologic toxicity precluded more intensive treatment in our group of patients. Second, the possible synergism between adriamycin and DTIC may best be exploited by administering adriamycin on the initial day of DTIC therapy.

LIST OF COMPOUNDS

Actinomycin D: NSC-3053
Adriamycin: NSC-123127; CAS reg. No. 23214-92-8
Cyclophosphamide: NSC-26271; CAS reg. No. 6055-19-2; 2*H*-1,3,2-oxazaphosphorine, 2-[bis(2-chloroethyl)amino]tetrahydro-, 2-oxide, monohydrate
DTIC: NSC-45388; CAS reg. No. 4342-03-4; imidazole-4-carboxamide, 5-(3,3-dimethyl-1-triazeno)-
Methyl-CCNU: NSC-95441; CAS reg. No. 33073-59-5; urea, 1-(2-chloroethyl)-3-(4-methylcyclohexyl)-1-nitroso-
Vincristine: NSC-67574; CAS reg. No. 2068-78-2; leurocristine, sulfate (1:1)

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Phase I and II Evaluation of Cytembena in Disseminated Epithelial Ovarian Cancers and Sarcomas[1,2]

Brief Reports and Preliminary Communications

Laurence H. Baker,* Michael K. Samson, and Ronald M. Izbicki[3]

Cytembena is a Czechoslovakian anticancer agent recently introduced into clinical trial in the United States. Cytembena has been reported to be active against ovarian cancer, cervical cancer, and disseminated sarcomas (1–6).[4] In the European and US studies thus far, cytembena has not appeared to be myelosuppressive thus making this agent attractive for further combination therapy. In an intermittent single-dose iv schedule by Moertel et al (7) an "autonomic storm" phenomenon became dose limiting. Henderson,[5] using a daily iv schedule, did not observe this sympathomimetic toxicity. Grafnetterová et al (8) reported that cytembena is tolerated by the im route; many European patients have been treated via this route. Since it would be much more practical to administer this agent in a protracted course via the im route, we therefore began a phase I study of patients with refractory ovarian cancers and sarcomas using as our initial dose that previously reported by the Europeans to be effective.

MATERIALS AND METHODS

Patients were accepted into our study if they had disseminated sarcomas or ovarian carcinomas refractory to or not eligible for more proven efficacious therapy or a protocol of higher priority. Upon entry into the study all patients must have had histologic proof of their malignancy and strong clinical evidence of dissemination. They must not have received any prior chemotherapy, radiotherapy, or surgery involving resection or anastomosis within 30 days of the start of cytembena. In addition, patients must have had demonstrable normal renal function as well as normal cardiac function as defined by history, physical examination, and noninvasive cardiology techniques. All patients must have had an expected survival time of ≥ 6 weeks and have signed a consent form in accordance with our institutional policy.

The initial drug dosage was 125 mg/m²/day given im for 20 days. Subsequent doses were 200, 300, and 400 mg/m². The treatment schedule was such that after the 20 daily im injections, a 10-day rest period was allowed and then another cycle of therapy was re-instituted. No patient was allowed to receive a dose escalation in order to observe any possible cumulative toxicities. Treatment was stopped if there was disease progression after two courses of therapy or if progression was > 25% after one course of therapy.

Twenty-one patients were entered into the study. One patient died 4 days after the start of treatment from her tumor. The remaining 20 patients were evaluable for response. There were ten patients entered with ovarian cancer with a stage 3 or 4 (FIGO) diagnosis, six patients had papillary cystadenocarcinomas, three patients had mucinous adenocarcinomas, and one patient had an endometrioid carcinoma of the ovary. Nine of the ten ovarian carcinoma patients had received prior chemotherapy. Each of the nine patients had received cyclophosphamide as a single agent or in combination. Six of these patients had received two or more prior chemotherapeutic drugs, including 5-fluorouracil, mitomycin C, and/or adriamycin. In addition, six of the patients had received cobalt 60 radiotherapy to the abdomen and/or pelvis. Ten patients with widely disseminated sarcomas were also treated with cytembena. Histologic diagnoses included neurofibrosarcoma in two patients and liposarcoma, synovial cell sarcoma, carcinosarcoma of the uterus, angiosarcoma, rhabdomyosarcoma, osteogenic sarcoma, fibrosarcoma, and leiomyosarcoma each in one patient. Nine patients had received prior chemotherapy consisting of a four-drug combination including

[1]Received May 25, 1976; revised Sept 7, 1976; accepted Sept 20, 1976.

[2]Cytembena: NSC-104801; CAS reg. No. 16170–75–5; acrylic acid, 3-*p*-anisoyl-3-bromo-, sodium salt. Supplied by the Cancer Therapy Evaluation Program (CTEP), Division of Cancer Treatment (DCT), National Cancer Institute (NCI).

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[4]Slavik M. Cytembena (NSC-104801). An updated review, 1975.

[5]Henderson E. Personal communication.

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adriamycin (cyclophosphamide, vincristine, adriamycin, and actinomycin D or cyclophosphamide, vincristine, adriamycin, and DTIC). Three of the patients also received cobalt 60 radiotherapy. In the 20 patients evaluable for toxic effects and response, the mean age was 51 years (range, 19–80 years). Eighteen of the patients were women and 13 were white.

RESULTS

Table 1 shows the number of patients and courses administered at the various dose levels. Unfortunately, no clinical responses could be observed in any of the 20 patients treated with cytembena.

The limiting toxic effect was pain at the injection site with increasing volumes of cytembena being administered. At the 300- and 400-mg/m^2 dose levels it became necessary to divide the daily dose into two injections. Three patients experienced sterile abscesses at the injection site. Nausea was experienced by four patients but in no patient could vomiting be attributed to drug administration. One patient described an iodine taste in her mouth shortly after injection (cytembena contains a bromide ion). Careful monitoring of peripheral blood cell counts did not reveal any myelosuppressive activity from cytembena. The mean lowest platelet count for the entire group was 285 $\times$ 10^3 platelets/mm^3 while the mean lowest wbc count was 7.5 $\times$ 10^3 cells/mm^3. No appreciable change in the hematocrit or hemoglobin levels could be observed. In seven of 12 patients that had serial chemistry determinations, a significant rise in serum alkaline phosphatase was observed. In only one patient was there a corresponding increase in SGOT and in all seven patients there was no evidence of metastatic liver or bone involvement at the start of therapy.

Renal function could be monitored serially in 18 patients. In six patients an abnormal elevation in serum creatinine, BUN, and proteinuria could be detected. One patient at autopsy had evidence of obstruction (tumor)-induced renal failure (creatinine, 7.7 mg/100 ml; BUN, 320 mg/100 ml; moderate proteinuria). The other five patients had mild alterations in function. Elevated creatinine levels ranged from 2.2 to 3.2 mg/100 ml. Elevated BUN levels ranged from 23 to 47 mg/100 ml. All patients with serum enzyme elevation had trace to moderate proteinuria. Three patients who had mild alteration in renal function received 200 mg/m^2, one patient received 300 mg/m^2, and another received 400 mg/m^2. The patient who died received 300 mg/m^2. We could not document any patient experiencing an "autonomic storm." Serial blood pressure determinations, electrocardiograms, and physical examinations revealed no significant change in any patient. Serial measurement of vanillymandelic acid and catecholamines in four patients revealed no significant abnormality.

Only seven of the 20 patients studied received 40 or more injections (two complete courses) of cytembena. Four patients died prior to completion of a second course while in nine patients the measurable disease had increased > 25% over the pretreatment measurements.

CONCLUSION

We deliberately chose to use the im schedule and this dose formulation in an attempt to imitate the Czechoslovakian studies. Although we used a schedule very analogous to that of Dvorak (4), we were unable to demonstrate any therapeutic activity against ovarian cancer. Similarly, we were unable to demonstrate any clinical benefit in soft tissue sarcomas. Our toxicity observations were generally consistent with those in the European and US studies.

We confirmed the lack of myelosuppression with these dosage schedules and did note that pain at the injection site became dose limiting when trying to increase the volume of drug administered. Like Nagel et al[6] we did notice a rise in the serum alkaline phosphatase. Whether or not this rise was a

[6]Nagel GA, Mayer AC, Schmid I, et al. A phase I study with the antitumor agent sodium-*cis*-β-4-methoxybenzoyl-β-bromoacrylate (MBBA). Preprint on file with the Investigational Drug Branch, CTEP, DCT, NCI.

TABLE 1.—Cytembena administration

Im dose level (mg/m^2/day $\times$ 20)	No. of courses	Total No. of patients	Tumor type (No. of patients)	
			Ovary	Sarcoma
125	9	5	3	2
200	10	9	4	5
300	5	3	1	2
400	3	3	3	

result of the drug administration or tumor progression could not be determined with certainty although clinical judgment suggests that the rise in alkaline phosphatase was a result of cytembena administration. Although cytembena is relatively free of disturbing side effects and would therefore be of potential value in combination therapy, we were unable to confirm clinical effectiveness of this agent in the patient population we treated. All patients demonstrated either increasing disease or died from their cancer.

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Combination Chemotherapy With Methyl-CCNU, 5-Fluorouracil, and Vincristine in Adenocarcinoma and Large Cell Carcinoma of the Lung[1,2]

Brief Reports and Preliminary Communications

Albert M. Bernath,* Martin H. Cohen, Daniel C. Ihde, Byron E. Fossieck, Mary J. Matthews, and John D. Minna[3]

Adenocarcinoma and large cell anaplastic carcinoma of the lung remain a therapeutic frustration. These cell types occur commonly (1) and are characterized by widely variable biologic behavior, frequent dissemination early in the course of disease (2), and resistance to radiotherapeutic and chemotherapeutic endeavors (3). Although occasional encouraging preliminary reports appear in the chemotherapeutic literature (4), no large studies have demonstrated significant survival benefit.

The improvement in response rate seen in the study by Moertel et al of advanced colorectal adenocarcinoma with the three-drug combination of methyl-CCNU, 5-fluorouracil (5-FU), and vincristine (5) prompted us to evaluate a similar combination in adenocarcinoma and large cell anaplastic carcinoma of the lung. Although the response rates to the individual agents in this combination have not been impressive in lung cancer (3,6,7), the lack of other efficacious single agents or drug combinations for these cell types has justified this phase II effort.

METHODS

Nineteen patients, two women and 17 men from 31 to 69 years of age, with histologically confirmed extensive adenocarcinoma or large cell anaplastic carcinoma of the lung were studied. "Extensive" disease refers to carcinoma extending beyond one hemithorax. The cell types were large cell anaplastic carcinoma (three patients) and adenocarcinoma (16 patients). All patients were ambulatory and, with one exception, had a Zubrod performance status of 2 or better. Eleven patients had had no prior therapy, six had received prior radiotherapy, and two had had prior chemotherapy (cyclophosphamide in one patient and adriamycin and procarbazine in the other). All patients had measurable disease. In those patients who had received prior chest radiotherapy there was measurable disease outside the radiation field or the radiated lesion had increased in size by ≥ 50% before the start of chemotherapy. All patients had negative upper gastrointestinal series, barium enemas, stool guaiacs, and iv pyelograms before the initiation of treatment. Serum acid phosphatase levels were normal and the prostatic examinations were negative in all male patients. All patients had wbc counts of > 4000 cells/mm^3 and platelet counts of > 100,000 platelets/mm^3. All of the patients gave their informed consent to participate in this study.

Drugs were administered in the following schedule: methyl-CCNU, 150 mg/m^2 orally every 6 weeks; 5-FU, 500 mg/m^2 iv every week; and vincristine, 1.0 mg/m^2 iv every 6 weeks. Dosage reductions for neutropenia and thrombocytopenia were generally designed to maintain wbc counts between 3000 and 4000 cells/mm^3 and platelet counts at > 75,000 platelets/mm^3. The criteria for tumor response and progression were: *complete response*—disappearance of all evidence of disease for at least 6 weeks (one cycle of chemotherapy); *partial response*—≥50% reduction in the product of the largest perpendicular diameters of the tumor persisting for ≥ 6 weeks; and *stable disease*—a minimal period of ≥ 6 weeks in which neither response nor progression (50% increase in the product of perpendicular diameters) could be documented.

RESULTS

Table 1 lists features of the drug treatment in this group of patients. The regimen was quite tolerable with respect to hematologic and other toxic effects. The most frequent reason for dosage reduction was thrombocytopenia (50% of the patients had platelet count nadirs ≤ 100,000 platelets/mm^3).

All of the 19 patients in our study were considered

[1]Received June 4, 1976; accepted July 9, 1976.

[2]Methyl-CCNU: NSC-95441; CAS reg. No. 33073-59-5; urea, 1-(2-chloroethyl)-3-(4-methylcyclohexyl)-1-nitroso-.

5-Fluorouracil: NSC-19893; CAS reg. No. 51-21-8.

Vincristine: NSC-67574; CAS reg. No. 2068-78-2; leurocristine, sulfate (1:1).

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evaluable for response. Although no patient in the study experienced a complete or partial response, two (both with adenocarcinoma) had minor responses with transient shrinkage of nodes and stabilization of chest disease for 8 and 20 weeks, respectively. Eight additional patients had stable disease for periods ranging from 8 to 13 weeks. Six of the eight patients with stable disease had adenocarcinoma and two had large cell carcinoma. Of the 15 patients who had progressive disease when this report was prepared, nine had progression of chest disease alone, four had simultaneous progression in the chest and other metastatic sites, and two had progression in remote metastases alone.

With seven of the 19 patients still alive, the median survival time for the group was 15.0 weeks from the first day of treatment (by life-table analysis). The median time to progression was 11.0 weeks.

DISCUSSION

It is uncertain if the poor response rate to methyl-CCNU, 5-FU, and vincristine seen in this phase II study reflects the fact that weekly 5-FU was used instead of the daily × 5 schedule employed by Moertel et al (5). Kisner et al (8) however, using a regimen identical to ours, reported a response rate of 43% in colorectal adenocarcinoma; this response rate is equal to that of Moertel et al. Characteristics of our patient population did not seem to prejudice the results since the patients had an overall good performance status with a median age of 55 years, and had received minimal prior therapy.

We conclude that this combination-drug treatment has insufficient activity to warrant further study in these lung cancer cell types.

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TABLE 1.—Summary of chemotherapy with methyl-CCNU, 5-FU, and vincristine

	Cycle 1	Cycle 2
No. of patients treated	19	12
Median % of projected doses received for—		
Methyl-CCNU	100	91
5-FU	92	82
Vincristine	100	97
Median wbc nadir in cells/mm³ (range)	4100 (1900–10,500)	4000 (2000–5600)
Median platelet nadir in cells × 10^3/mm³ (range)	100 (35–218)	140 (83–270)
No. of patients (%) with—		
Vomiting	6 (32)	5 (42)
Paresthesias	1 (5)	2 (17)
Mucositis	2 (11)	0

Phase II Study of ICRF-159 in Patients With Metastatic Colorectal Carcinoma Previously Exposed to Systemic Chemotherapy[1,2,3]

Brief Reports and Preliminary Communications

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ICRF-159, a bisdiketopiperazine, was originally synthesized by the Imperial Cancer Research Fund facilities in London, England. This non-polar derivative of ethylenediaminetetraacetic acid has manifested significant antitumor activity against both early and advanced murine L1210 leukemia (1). Phase I evaluation of ICRF-159 in man (2) determined a maximum tolerated oral dose of 1 g/m^2/day for 3 days given in divided doses every 8 hours. During that study, one patient with metastatic colorectal carcinoma experienced an objective remission of 4 months' duration. On that basis, we initiated a phase II evaluation of ICRF-159 in patients with metastatic colorectal carcinoma refractory to 5-fluorouracil (5-FU)[8] systemic chemotherapy.

MATERIALS AND METHODS

ICRF-159 was administered to 38 patients with advanced, measurable metastatic colorectal carcinoma. Histologic confirmation of metastatic disease was required for entry into the study. Patients with a life expectancy of < 3 months or who had had systemic chemotherapy within 28 days of the initiation of the study were ineligible for treatment. Patients with a hemoglobin value of < 9 g/100 ml, wbc count < 4000 cells/mm^3, platelet count < 100,000 platelets/mm^3, or BUN > 25 mg/100 ml were also ineligible. Patients with a serum bilirubin > 1.5 mg/100 ml or an SGOT > 120 IU were excluded from this trial. The patient population included 21 men and 17 women. The mean age was 59 years (range, 35–75 years) for men and 59 years (range, 42–75 years) for women. All patients had previously received systemic chemotherapy with 5-FU. In addition, six patients had been treated with methyl-CCNU[9] as a single agent. Two men and one woman had achieved partial remissions of 4.0, 3.8, and 3.5 months' duration as a consequence of therapy with 5-FU. No patient treated with methyl-CCNU experienced an objective response.

ICRF-159 was administered orally at a dosage of 1 g/m^2/day for 3 consecutive days in divided doses every 8 hours. Courses of therapy were repeated at 21-day intervals until measurable progression (> 25% increase in tumor volume) was demonstrated. Palpable dermal, subcutaneous, lymph node, and intra-abdominal mass lesions were measured every 3 weeks. Pulmonary parenchymal lesions were evaluated with chest roentgenograms at 3-week intervals. Hepatic metastatic disease was evaluated by liver scan at 6-week intervals.

Patients were considered evaluable for toxic effects and response if there was a minimum followup of 14 days after the initiation of systemic chemotherapy. There were three response categories: *complete remission*—regression of all measurable lesions resulting in no evidence of disease; *partial remission* (PR)—a > 50% decrease in the sum of the products of the perpendicular diameters of all measurable disease for ≥ 1 month; and *no response*—all other patients evaluable for response.

Hemoglobin determinations and wbc and platelet counts were performed on Days 1, 14, and 21 of each treatment cycle. BUN, total serum bilirubin, SGOT,

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[2]Supported in part by Public Health Service grants CA-13456, CA-06927 (National Cancer Institute [NCI]), and RR-05539 (Division of Research Resources) from the National Institutes of Health, Department of Health, Education, and Welfare; and by an appropriation from the Commonwealth of Pennsylvania.

[3]ICRF-159; NSC-129943; CAS reg. No. 21416-87-5; 2,6-piperazinedione, 4,4'-propylenedi-, (±)-. Supplied by the Cancer Therapy Evaluation Program, Division of Cancer Treatment, NCI.

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[8]5-Fluorouracil: NSC-19893; CAS reg. No. 51-21-8.

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[9]Methyl-CCNU: NSC-95441; CAS reg. No. 33073-59-5; urea, 1-(2-chloroethyl)-3-(4-methylcyclohexyl)-1-nitroso-.

TABLE 1.—Toxic effects of ICRF-159 in 35 evaluable patients*

Toxic effect	No. of patients
Gastrointestinal	15
Nausea	15
Emesis	11
Diarrhea	2
Hematologic	16
Leukopenia (< 2000 cells/mm³)	12
Thrombocytopenia (< 100,000 platelets/mm³)	4
Anemia	3
Alopecia	14
Flu-like syndrome	2
Headache	1

*1st course of therapy.

and alkaline phosphatase determinations were performed initially and at 21-day intervals.

RESULTS

Of 38 patients treated with ICRF-159, 35 were evaluable for toxic effects and response. Three patients died of metastatic disease within 14 days of initiation of therapy and were therefore not evaluable.

TOXICITY

Of the 35 patients evaluable for toxicity (table 1), 15 had nausea and 11 of these patients experienced a mild degree of emesis. Two patients had diarrhea.

Overall, a 67.8% (mean) reduction in the wbc count was noted on Day 14 with recovery to pretreatment values by Day 21 of the first treatment cycle; 12 patients had leukopenia of < 2000 cells/mm³. There was a 32.7% (mean) decrease in the platelet count on Day 14 with recovery by Day 21 of the first course of therapy; four patients experienced thrombocytopenia of < 100,000 platelets/mm³. In no case was there evidence of sepsis or a hemorrhagic diathesis. Three patients had anemia on the basis of bone marrow suppression which necessitated a transfusion of packed rbcs. Fourteen patients experienced significant alopecia. Two patients experienced a flu-like syndrome consisting of fever, chills, myalgias, and coryza during the 3 days of ICRF-159 administration. One patient complained of severe headaches during the administration of ICRF-159. There was no evidence of hepatic, pulmonary, renal, or cardiac toxic effects in any patient treated with ICRF-159. No ICRF-159 drug-related deaths were noted.

RESPONSE

Of 35 patients evaluable for response, PRs were induced in five patients treated with ICRF-159. All patients experiencing objective remission did so during the first course of treatment. Table 2 details the clinical data relative to the five patients responding to ICRF-159 chemotherapy. No ICRF-159 responder had previously experienced tumor regression as a consequence of systemic chemotherapy with either 5-FU or methyl-CCNU. The three previous responders to 5-FU failed to respond to therapy with ICRF-159. There was no relationship between ICRF-159-induced myelosuppression and antitumor activity. The remission duration was 4.0 (median) and 3.2 months (mean) with a range of 1.0–5.0 months.

The survival time from the initiation of ICRF-159 systemic chemotherapy was 6.0 (median) and 6.0+ months (mean) with a range of 4.0–8.0+ months for objective responders and 3.0 (median) and 4.8+ months (mean) with a range of 1.0–19.0+ months for nonresponders.

DISCUSSION

A phase I evaluation of ICRF-159 (2) revealed a maximum tolerated oral dose of 1 g/m²/day adminis-

TABLE 2.—Clinical summary of patients responding to ICRF-159 systemic chemotherapy

Patient No.	Sex	Age	Prior treatment*	Prior chemotherapy	Responding lesions	Remission duration (mos)	Survival (mos)
1	M	51	S,C	5-FU	Lung	4.0	4.0
2	M	54	S,R,C	5-FU Methyl-CCNU	Soft tissue	4.0	7.0
3	M	70	S,C	5-FU	Lymph nodes	1.0	8.0+
4	F	51	S,C	5-FU	Abdominal	5.0	6.0
5	F	61	S,C	5-FU	Lung	2.0	5.0

*S = surgery; R = radiation therapy; C = systemic chemotherapy.

tered for 3 consecutive days in divided doses every 8 hours. During that study, one of four patients with metastatic colorectal carcinoma responded to ICRF-159 systemic chemotherapy. The paucity of active single agents for the treatment of metastatic colorectal carcinoma led us to the present phase II trial of ICRF-159 in patients refractory to 5-FU systemic chemotherapy.

The toxicity manifested in our trial is consistent with the toxicity data reported in earlier studies (2–4).

Five clinically useful PRs were induced in 35 evaluable patients who received ICRF-159 in therapeutic doses. Marciniak et al (4) reported that no objective remissions resulted from ICRF-159 systemic chemotherapy in 12 patients with metastatic colorectal carcinoma who were previously exposed to systemic chemotherapy; three of 13 patients receiving ICRF-159 as primary systemic treatment experienced objective remissions.

A 14.3% objective response rate for ICRF-159 compares favorably with response rates reported for 5-FU and methyl-CCNU as single agents (5). ICRF-159 thus joins 5-FU and methyl-CCNU as "single agents with some degree of therapeutic activity" (5) in the treatment of advanced colorectal carcinoma. A prospective randomized phase III trial comparing 5-FU and ICRF-159 is warranted.

The occurrence of five responses in 35 evaluable patients indicates that, within 95% confidence limits, the true response rate for ICRF-159 in previously treated patients with metastatic colorectal carcinoma is between 4.8% and 30.3% (6). Further phase II evaluation of ICRF-159 in this class of patients would better define the true response rate.

Finally, Wampler et al (7) demonstrated antitumor synergism when ICRF-159 and 5-FU were combined in a murine L1210 leukemia system. A phase I–II evaluation of the combination of ICRF-159 plus 5-FU in metastatic colorectal carcinoma is therefore recommended.

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LETTERS

Combination Therapy With Mitomycin C, 5-Fluorouracil, and Cytosine Arabinoside in P815 Ascites and Solid Mouse Neoplasms[1,2]

In anticipation of a clinical trial with mitomycin C, 5-fluorouracil (5-FU), and cytosine arabinoside (MFC), we undertook a re-evaluation of the animal experiments of Hoshino et al (1) who first described this combination in L1210 mouse neoplasm. It should be pointed out that Hoshino's experiments were performed not with the solid tumor, but only with the ascites form of L1210, whereas advanced, solid tumors of the gastrointestinal tract were subjected to the MFC combination therapy in the clinical trial by De Jager et al (2).

In order to elucidate the influence of the location of the neoplasm upon the response to therapy, animal experiments were performed in which ascitic and solid P815 neoplasms were investigated. Also, the L1210 neoplasm lines of different laboratories (originally derived from the same L1210 line of the Sloan-Kettering Institute) were tested for response to the same dosage schedule and regimen.

About 10^6 P815 cells were injected ip or sc to groups of six female BDF$_1$ mice. The treatment, initiated 24 hours after the inoculation of the tumor, consisted of ten daily ip injections of: (*a*) 0.67 mg/kg of mitomycin C; (*b*) 8.3 mg/kg of 5-FU; (*c*) 6.7 mg/kg of cytosine arabinoside; or the combinations of a + b, a + c, b + c, and a + b + c. Control mice received saline only. The results of a representative experiment are given in table 1.

There is a clear-cut difference in the response of ascites versus solid tumor forms to the same drug(s) measured by an increase in lifespan (ILS). Mitomycin C and 5-FU (but not cytosine arabinoside) used singly showed considerably better responses in the ascitic than in the solid tumor form. All three combinations of two drugs resulted in even greater ILSs in the ascites than in the solid tumor form. The combination of mitomycin C and 5-FU was about ten times greater in the ascites than in the solid tumor. The three drugs in combination still favored the ascites tumors by a factor of two. Apparently, the direct contact of the cells with the drugs in the case of the ascites was much more

[1]Supported in part by Public Health Service grants CA-08748, CA-15857, and CA-18557 from the National Cancer Institute, National Institutes of Health, Department of Health, Education, and Welfare.

[2]Mitomycin C: NSC-26980; CAS reg. No. 50-07-7.
5-Fluorouracil: NSC-19893; CAS reg. No. 51-21-8.
Cytosine arabinoside: NSC-63878; CAS reg. No. 69-74-9; cytosine, 1-β-D-arabinofuranosyl-, monohydrochloride.

TABLE 1.—Effect of mitomycin C, 5-FU, and cytosine arabinoside either alone or in combinations of 2 or 3 in P815 neoplasms in ascites or solid tumor form in BDF$_1$ mice

		P815							
		Ascitic tumor				Solid (sc) tumor			
Treatment	Dose (mg/kg)	MST* (days)	%ILS	45-day survivors	Change in weight, Day 6 (g/mouse)	MST* (days)	%ILS	45-day survivors	Change in weight, Day 6 (g/mouse)
Control, saline		7		0	+3.3	8		0	+0.8
Mitomycin C	0.67	11	+57	0	−0.5	9	+13	0	0
5-FU	8.3	17	+143	0	+0.3	9	+13	0	+0.7
Cytosine arabinoside	6.7	14	+100	0	−0.2	14	+75	0	+0.5
Mitomycin C + 5-FU	0.67 + 8.3	33	+370	1	−1.0	11	+38	0	−0.7
Mitomycin C + cytosine arabinoside	0.67 + 6.7	33	+370	2	−0.5	20	+150	0	−0.5
5-FU + cytosine arabinoside	8.3 + 6.7	28	+300	1	−0.2	21	+166	0	−0.3
MFC	0.67 + 8.3 + 6.7	39	+457	2	−0.7	26	+225	1	−0.1

*Median survival time.

efficient than the contact with the drugs mediated by the bloodstream in the solid tumor cells. However, the quantitative difference between direct and indirect drug action varied greatly depending on the drug or drug combinations used. This might be ascribed to the different half-lives in plasma versus ascites fluid and to the different rates of metabolism of the drugs in these two body fluids.

No toxicity in the form of significant weight loss was observed in any of the drug regimens used in table 1.

When the results of the P815 ascites neoplasms are compared with the results of Hoshino et al (1) with the same treatment of L1210 ascites-bearing mice, we see comparable response in all regimens studied although the combination of the three drugs in the P815 line is somewhat, but probably not significantly, lower than the response of Hoshino's L1210 line (ILS, 457% versus 522%) (1). The difference was more pronounced with respect to long-term survivors (Hoshino's study, four of five 60-day survivors; our study, two of six 45-day survivors).

The L1210 lines, kindly supplied by Drs. J. H. Burchenal (NY) and A. Hoshino (Japan), were compared with that from Dr. Hutchison's Laboratory (Rye). All three lines were derived from the same L1210 line from Dr. Hutchison's laboratory and were carried independently in BDF_1 mice in each of these laboratories for several years. When these three L1210 lines were compared simultaneously in BDF_1 mice (ascitic form) for response to the MFC combination at the same dosages used in the experiments with P815 cells (table 1), the ILSs were similar in all three lines (Rye, +310%; NY, +200%; and Japan, +225%). Only the Rye line showed two 45-day survivors. These ILS values are below those in P815 ascites neoplasm and below the values originally reported for L1210 by Hoshino (1). Also, the MFC combination was toxic (by weight loss on Day 6) in the Rye line (−2.5 g/mouse) and close to toxic in the two other lines (−1.8 g/mouse).

From these animal experiments it can be concluded that the relatively poor response in patients with the combination of MFC therapy might well be due to the different tumor locations, drug supply, metabolism, and, mostly, response of the human cancer versus the L1210 cells. Apparently the results of Hoshino et al can be reproduced best in our P815 line, whereas our L1210 line as well as the other two lines of the same origin did not show results comparable to those originally reported by Hoshino et al (1). The cause is unclear, but might be due to the host BDF_1 mouse, seasonal differences in the response, different diet, or environmental factors.

Thus, it appears that the results of experimental drug combination studies, particularly with ascites tumors, are not always pertinent for the use of such combinations clinically. The solid experimental tumor system might be more relevant.

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July 1, 1976

Symptomatic Hypophosphatemia Secondary to 5-Azacytidine Therapy of Acute Nonlymphocytic Leukemia

5-Azacytidine, a pyrimidine nucleotide analog, is an effective chemotherapeutic agent in some cases of acute leukemia resistant to conventional drugs (1,2). We would like to describe the development of profound symptomatic hypophosphatemia in two leukemic patients following therapy.

CASE REPORTS

Patient No. 1

A woman developed acute myelogenous leukemia (AML) in her fifth month of pregnancy. Remission was induced with 6-thioguanine, daunorubicin, cytosine arabinoside, and prednisone. Cytosine arabinoside was discontinued due to development of severe exfoliative dermatitis. Treatment was continued with 6-thioguanine, daunorubicin, vincristine, and prednisone. A healthy full-term infant was delivered. Recurrent disease developed 2 months postpartum and BCNU and cyclophosphamide were administered without response. A course of 5-azacytidine was begun (150 mg iv every 8 hours), however, this therapy was complicated by severe generalized

[3]The author is indebted to Dr. D. J. Hutchison for her continuous interest in this study and to Drs. J. H. Burchenal and H. Hoshino for their L1210 tumor lines. The technical assistance of Cathy Hession and Doreen Leonard is also acknowledged.

muscular aching and tenderness. There was no demonstrable muscle weakness or arthritis. Pain and tenderness involved the tongue, as well as throat and jaw muscles, resulting in dysphagia. The patient had difficulty rising from a sitting position and she developed a shuffling gait because of pain associated with movement of the legs. The remainder of the physical examination was negative. A diagnosis of a drug-induced myositis was made. Serum phosphorus was 0.6 mg/100 ml (normal range, 3.5–5.0 mg/100 ml). The serum phosphorus level gradually returned to normal over the following 8 days (fig 1), and muscular symptoms abated. 5-Azacytidine in the same dosage was administered during two further courses of therapy. Hypophosphatemia occurred on each occasion (fig 1) and was accompanied by symptoms of muscle pain and tenderness. Only slight increases in 24-hour urine phosphorus excretion (maximum 834 mg/24 hours) were observed. Serum calcium and uric acid levels remained stable. Subtle and transient increases in the BUN level (7→21 mg/100 ml) and serum creatinine (0.5→0.9 mg/100 ml) were noted during the courses of therapy, however, daily urinalyses were normal and serial 24-hour urine protein excretions remained < 100 mg. Serial parathyroid hormone levels did not change. Further serial laboratory values obtained which were normal included determinations of SGOT, LDH, CPK, serum proteins, serum electrolytes, serum magnesium, 24-hour urine magnesium, and rbc adenosine triphosphate (ATP) and 2,3-diphosphoglycerate (2,3-DPG). Although a short-lived PR of AML was obtained with 5-azacytidine therapy, recurrent disease developed and the patient died in July 1975.

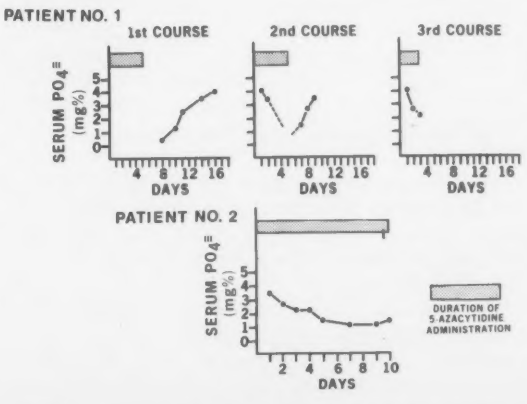

FIGURE 1.—Serum phosphorus levels during treatment with 5-azacytidine.

Patient No. 2

A 40-year-old Oriental man developed AML. Remission was induced with daunorubicin, 6-thioguanine, and cytosine arabinoside. An initial relapse was treated with cyclophosphamide, vincristine, cytosine arabinoside, and prednisone, a second relapse with these agents plus immunotherapy with BCG and allogeneic leukemia cells, and a final relapse with 5-azacytidine at 100 mg/day iv. As in the previous patient, therapy with the latter drug was complicated by severe muscle cramps, aching, and tenderness without demonstrable muscle weakness. These symptoms were again accompanied by the development of hypophosphatemia (fig 1). No significant changes occurred in 24-hour urine phosphorus, calcium, uric acid, or protein excretion. BUN, serum creatinine, electrolytes, calcium, and uric acid levels remained stable. 5-Azacytidine therapy was complicated by the development of leukopenia and thrombocytopenia and the patient died of an intracranial hemorrhage on the 10th day of treatment.

To summarize, severe muscle pain and tenderness developed during four courses of 5-azacytidine therapy in two patients with AML. Although hypophosphatemia may have led to muscle symptoms secondary to a depletion of high-energy phosphate compounds or to a decrease in rbc 2,3-DPG, assays of rbc ATP and 2,3-DPG in one patient were normal. For the present, therefore, the exact basis of the muscular symptoms, although seemingly related to the hypophosphatemia, remains to be determined. It is of interest, however, that muscular symptoms of undefined origin have previously been described in patients receiving 5-azacytidine therapy (3).

The genesis of the hypophosphatemia remains unclear at the present time. In each case, 24-hour urine phosphorus excretion was documented and remained relatively stable. Furthermore, in these patients severe gastrointestinal (GI) toxicity with vomiting and diarrhea did not occur. It is possible, therefore, that 5-azacytidine influences the GI absorption or intracellular migration of phosphorus. These and other aspects of phosphorus metabolism should be studied further in patients receiving 5-azacytidine therapy.

LIST OF COMPOUNDS

5-Azacytidine: NSC-102816; CAS reg. No. 320–67–2; *s*-triazin-2(1*H*)-one, 4-amino-1-β-D-ribofuranosyl-

BCNU: NSC-409962; CAS reg. No. 154–93–8; urea, 1,3-bis(2-chloroethyl)-1-nitroso-

Cyclophosphamide: NSC-26271; CAS reg. No. 6055–19–2; 2*H*-1,3,2-oxazaphosphorine, 2-[bis(2-chloroethyl)amino]tetrahydro-, 2-oxide, monohydrate

Cytosine arabinoside: NSC-63878; CAS reg. No. 69–74–9; cytosine, 1-β-D-arabinofuranosyl-, monohydrochloride

Daunorubicin: NSC-82151; CAS reg. No. 23541-50-6
Prednisone: NSC-10023; CAS reg. No. 53-03-2; pregna-1,4-diene-3,11,20-trione, 17,21-dihydroxy-
6-Thioguanine: NSC-752; CAS reg. No. 1832-72-0; purine-6-thiol, 2-amino-
Vincristine: NSC-67574; CAS reg. No. 2068-78-2; leurocristine, sulfate (1:1)

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July 15, 1976

Pigmentation of the Tongue After Treatment With Adriamycin

Adriamycin is an antitumor antibiotic with a wide range of activity against several malignant neoplasms in children and adults (1,2). The toxic effects of adriamycin consist of hematologic depression, alopecia, stomatitis, gastrointestinal disturbances, electrocardiographic changes, and cardiomyopathy (1). Hyperpigmentation of the skin, nails, and nailbeds has been reported after the administration of adriamycin (2–4). The purpose of this paper is to report the development of hyperpigmentation of the tongue in two children after adriamycin therapy.

CASE REPORTS

Patient No. 1.—A 9-year-old black girl had a left nephrectomy at 6 years of age because of a Wilms' tumor. After the surgery she received radiotherapy and chemotherapy, the latter consisting of vincristine and actinomycin D for 6 months, followed by actinomycin D alone for an additional 18 months. One year after the discontinuation of chemotherapy, pulmonary metastasis was detected for which she received irradiation to both lung fields (1400 rads) followed by chemotherapy with adriamycin and vincristine. Adriamycin was given at a dose of 20 mg/m²/day for 3 days and repeated every 6 weeks, and vincristine was given at a dose of 1.5 mg/m²/week for 4 weeks and repeated every 3 months.

Two weeks after the start of the second, fourth, and fifth courses of adriamycin, multiple black pigmented lesions, some with papillary projections, were noted over the tongue (fig 1). No such lesions were noted over the buccal mucosa. However, the patient also received a dose of vincristine on the first day of the second and fourth courses of adriamycin but none with the fifth. The microscopic examination of scrapings from the tongue lesions revealed candida. The tongue lesions faded gradually over the next 2–4 weeks. Moderate black pigmentation of the skin over the interphalangeal joints and the nailbeds was also observed which has remained unchanged during the treatment period.

Patient No. 2.—A 19-month-old black girl with acute lymphoblastic leukemia went into remission with the combination of vincristine and prednisone. After this combination she received central nervous system prophylaxis with cranial irradiation and intrathecal methotrexate. After 3 months of continuous chemotherapy with methotrexate, 6-mercaptopurine, and cyclophosphamide she had a bone marrow relapse. Because of some problems encountered during the initial remission induction with vincristine and prednisone, adriamycin (30 mg/m²/day × 2 days) was used for remission induction. Two weeks after the first and second courses of adriamycin at 4-week intervals, flat black pigmented lesions were noted over the tongue which cleared over 2 weeks.

COMMENT

Pigmentation of the skin, nails, and nailbeds has been reported after the administration of adriamycin (2–4). Pratt and Shanks (3) have reported such changes in 11 of 52 children (five of 13 black and six of 39 white) receiving adriamycin. Pigmentation of the tongue was not observed in any of the children. O'Bryan et al (2) reported skin pigmentation in two of 472 patients following adriamycin therapy. Finally, Rothberg et al (4) have described marked black pigmentation of the palms, soles, nailbeds, and skin over the dorsa of the knuckles, and several small hyperpigmented areas on the buccal mucosa in a 70-year-old black woman after the administration of adriamycin. A biopsy of the hyperpigmented skin from the back of the knuckle showed increased melanin deposition.

We have noted pigmentation of the tongue in two of ten children (two of eight black and none of two nonblack) receiving adriamycin. The response to adriamycin could not be judged in five patients with solid tumors because they received other chemotherapeutic agents simultaneously. Of the five chil-

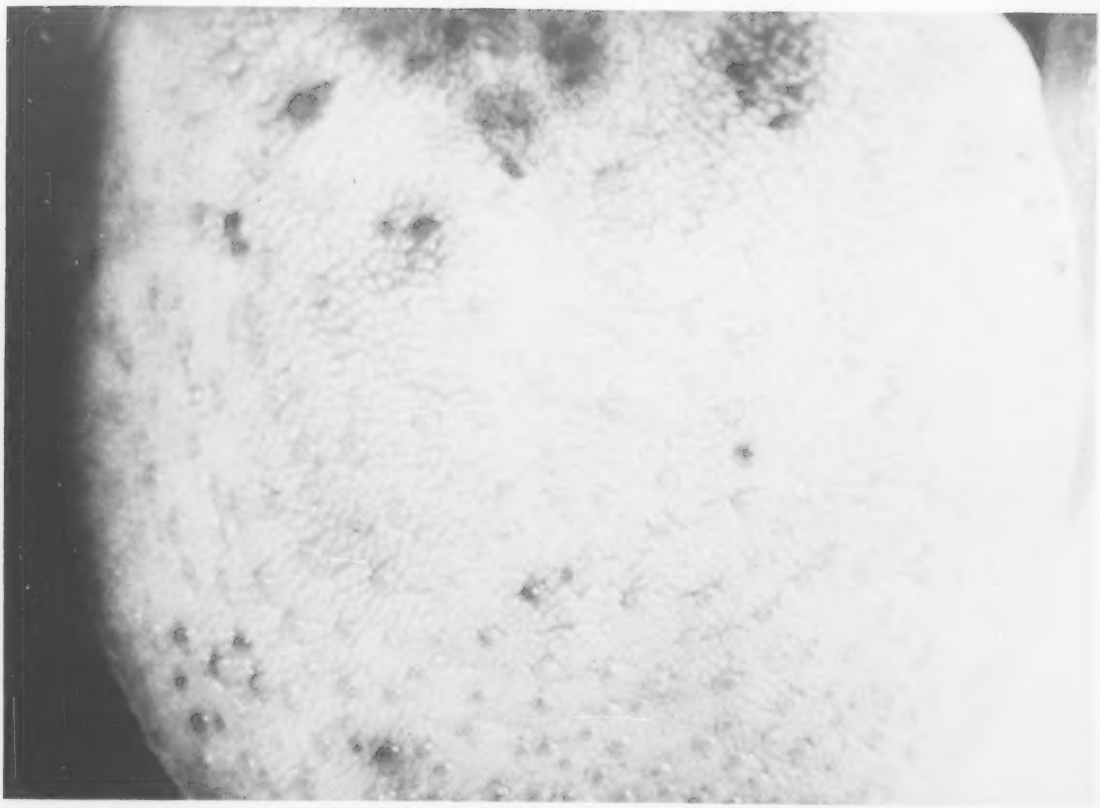

FIGURE 1.—Black pigmentation of the tongue in patient No. 1, 2 weeks after the adriamycin administration.

dren with acute leukemia receiving adriamycin alone for remission induction, two had CR, two had PR, and one did not respond. In general, the remission duration was short.

In both of our patients there was an interval of 2 weeks between administration of the medication and appearance of pigmented lesions. These two case reports suggest that adriamycin may have been the cause of tongue pigmentation in each patient, that the pigmentation appears to be reversible, and that in our experience the effect is confined to black children.

LIST OF COMPOUNDS

Actinomycin D: NSC-3053

Adriamycin: NSC-123127; CAS reg. No. 23214-92-8

Cyclophosphamide: NSC-26271; CAS reg. No. 6055-19-2; 2*H*-1,3,2-oxazaphosphorine, 2-[bis(2-chloroethyl)amino]tetrahydro-, 2-oxide, monohydrate

6-Mercaptopurine: NSC-755; CAS reg. No. 6112-76-1; purine-6-thiol, monohydrate

Methotrexate: NSC-740; CAS reg. No. 59-05-2; glutamic acid, *N*-[*p*-[[(2,4-diamino-6-pteridinyl)methyl]methylamino]benzoyl]-

Prednisone: NSC-10023; CAS reg. No. 53-03-2; pregna-1,4-diene-3,11,20-trione, 17,21-dihydroxy-

Vincristine: NSC-67574; CAS reg. No. 2068-78-2; leurocristine, sulfate (1:1)

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July 26, 1976

ANNOUNCEMENTS

Annual Meeting of the American Association for Cancer Research, Inc

Denver, Colorado, May 18–21, 1977

The scientific sessions of the 68th Annual Meeting of the American Association for Cancer Research, Inc, will be held at the Denver Hilton Hotel, Denver, Colorado, from 8:30 AM, Wednesday, May 18, until 5:00 PM, Saturday, May 21, 1977. This meeting will be preceded by that of the American Society of Clinical Oncology to be held in the same hotel on May 16 and 17, 1977.

Registration for the Association meeting will be conducted only at the Denver Hilton Hotel and will take place during the afternoon and evening of Tuesday, May 16, 1977, and throughout the course of the Annual Meeting. Nonmembers may obtain programs and purchase abstracts of the papers (the Proceedings) at the time of registration. By action of the Board of Directors, a registration fee of $20.00 will be charged each member and nonmember attending the scientific sessions; a special rate of $5.00 will be charged predoctoral students who present a statement, signed by the registrar, dean, or department head of their university or college, confirming their status. Admission to the scientific sessions will be restricted to properly registered persons.

A copy of the detailed regulations involving submission of abstracts and presentation of papers will be sent to each active member of the Association as part of the customary Annual Letter to be issued in October 1976. Corresponding members and nonmembers may obtain copies of the regulations and hotel reservation cards from the Secretary-Treasurer. The deadline for receipt of abstracts, December 15, 1976, will be enforced strictly. Abstracts that are submitted in conformity with the regulations and considered of acceptable quality will be published in the March 1977 Proceedings of the American Association for Cancer Research, which will be mailed by the publisher to all paid-up subscribers to the journal *Cancer Research*.

For further information contact: Hugh J. Creech, Secretary-Treasurer, American Association for Cancer Research, Inc, The Institute for Cancer Research, Fox Chase, Philadelphia, Pa 19111.

Johananoff International Fellowship, 1977–1978

Applications are invited for the 1977–1978 Johananoff International Fellowship for Advanced Biomedical Studies which is awarded by the Mario Negri Institute Foundation Inc, New York, and the Mario Negri Institute for Pharmacological Research, Milan, Italy. The fellowship, amounting to $15,000 for 1 year, was established in 1974 through the far-sightedness and generosity of Mr. S. Johananoff, a distinguished international industrialist.

The Johananoff Fellow will spend a sabbatical year at the Mario Negri Institute in Milan free from laboratory, administrative, and other routine duties, in order to plan an advanced study critically reviewing achievements in one of the following fields: cancer chemotherapy and/or immunology, cardiovascular pharmacology, neuropsychopharmacology, or drug metabolism.

Candidates for the fellowship should be non-Italian, mid-career scientists, internationally known for their work on one of the stated topics. Applications should include a curriculum vitae, list of publications, and outline of proposed studies and must be received in Milan by January 31, 1977. *Requests for application kits should be airmailed to:* The Johananoff Fellowship Committee, Istituto di Ricerche Farmacologiche Mario Negri, Via Eritrea 62, 20157 Milan, Italy (Tel. [02] 355 4546 Telex 37268 NEGRINST).

The Continuing Medical Education Institute Course on "The Changing Seasons of Life: The Physician's Role"

S. S. Rotterdam, New York to Nassau and Bermuda, April 23–30, 1977

The Continuing Medical Education Institute course on "The Changing Seasons of Life: The Physician's Role" to be held aboard the S. S. Rotterdam (New York to Nassau and Bermuda) April 23–30, 1977, is being sponsored by Sea and Shore Seminars, Washington, DC, the Charles R. Drew Postgraduate Medical School, Office of Continuing Education, and the University of Cincinnati, College of Medicine, Office of Continuing Medical Education. The registration fee is $175.00 (after February 23, 1977, the fee will be $200.00).

Medical problems of interest to both generalists and specialists will be presented in a continuum, prenatal through geriatric years. The course is acceptable for 25 credit hours in Category I for the Physician's Recognition Award of the American Medical Association. Additional professional areas for which accreditation will be available will be sent on request.

For further information contact: **Sea and Shore Seminars, 2100 19th St, NW, No. 601, Washington, DC 20009 (Attn: N. Chamberlain).**

CORRECTION

We regret that an error appeared on page 744 of the June 1976 issue of *Cancer Treatment Reports* ("Clinical Trials With BCNU (NSC-409962) in Malignant Lymphomas by the Eastern Cooperative Oncology Group" by John M. Bennett et al). The dose for prednisone in the COPP and CP regimens (table 8) should be 100 mg/m^2/day orally × *5* days, not × *14* days as printed.

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